RNAi of selected insect genes

By

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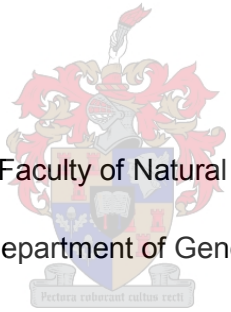
Magister Scientiae



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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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December 2016

Abstract

Diuraphis noxia (Kurdjumov, Hemiptera: Aphididae), commonly known as the Russian wheat aphid (RWA), is regarded as one of the most destructive and widely distributed insect species in the world. Nonetheless, the currently available control strategies, including chemical pesticides, biological control agents, and RWA resistant wheat cultivars, are still very limited and rather ineffective. The process of double-stranded RNA (dsRNA)-mediated interference (RNAi) displays high specificity and the prospect of developing into a new specific method for managing agricultural pests. Plants can potentially be genetically engineered to express dsRNA to down-regulate vital gene functions present in pest insects, resulting in the protection of plants. In order to survive and reproduce, aphids require close interaction with their host plants, during which effectors are transported inside the plant to modify host cell processes. Four previously identified RWA salivary secretion proteins were investigated in the present study. However, cloning and sequencing results indicated that only two of the aforementioned proteins – C002 and 14-3-3 epsilon (ε) – could be potential protein elicitors in RWA. Thus, these two transcripts were subjected to RNAi experiments via artificial diet feeding and feeding on siRNA injected wheat leaf trials in order to investigate their role in RWA-host interactions and their importance in the survival and reproduction of the RWA. The relative expression levels of *C002* and *14-3-3 ε* at 0h were compared between SAM, the most virulent RWA biotype, and SA1, the least virulent RWA biotype in South Africa, and the results indicated that both transcripts had a higher relative expression in SAM than in SA1. Therefore, suggesting that *C002* and *14-3-3 ε* might play an important part in RWA virulence. From the RT-qPCR results it was evident that successful silencing of both *C002* and *14-3-3 ε* were achieved at 24h after initial siRNA exposure and that the transient silencing effect subsided thereafter. The expression data pertaining to the wheat leaf injection experiments, however, displayed high standard deviations that are not ideal and suggested that the expression of the transcripts differs greatly between the aphids within each group. This is likely due to the custom-made aphid cages and injection procedure of the siRNA into wheat leaves that appears to hinder the accuracy of the results. The fecundity data produced quite inconclusive results due to previously mentioned

inadequacies and therefore an accurate and decisive conclusion cannot be drawn as to how the *C002* and *14-3-3 ε* silencing effects the survival and reproduction of the RWA. Both methods used for RNAi – the artificial diet trial and the injection of wheat leaves trial – have their drawback. After considering the RT-qPCR data, it appears as though the artificial diet trial produced more accurate and feasible results. Even so, the injection method establishes a more natural mode of feeding for the aphids and consequently more optimal cages need to be designed and tested to produce precise results.

Uittreksel

Diuraphis noxia (Kurdjumov, Hemiptera: Aphididae), wat algemeen bekend staan as die Russiese koringluis (RWA), is een van die mees vernietigende en wydverspreide insekspesies ter wêreld. Daarteenoor is die huigdige beskikbare beheer-strategieë, wat chemiese plaagdoders, biologiese beheermiddels, en RWA-weerstandige koringkultivars insluit, steeds baie beperk en grootendeels oneffektief. Die proses van dubbelstring-RNA (dsRNA)-gemedieerde inmenging (RNAi) toon hoë spesifisiteit en die vooruitsig om te ontwikkel in ‘n nuwe spesifieke metode vir die beheer van landboukundige peste. Plante kan potensieël genetiese engineerswese ondergaan om dsRNA uit te druk om die afregulering van noodsaaklike geenfunksies in insekpeste te bewerkstellig en sal lei tot die beskerming van plante. Vir koringluise om te oorleef en voort te plant, benodig hul nabye interaksie met hul gasheerplante waartydens effektore in die plant vervoer word om die gasheerselle se prosesse te verander. Vier voorheen geïndifiseerde RWA-speeksel sekresie-proteiëne is in die huidige studie ondersoek. Die resultate van die klonering en volgorderbepaling het egter getoon dat slegs twee van die proteiëne – C002 en 14-3-3 epsilon (ε) - moontlike proteiën-elisitore in die koringluis is. Dus is hierdie twee transkripte blootgestel aan RNAi-eksperimente, deur gebruik te maak van ‘n kunsmatige dieet benadering asook deur koringblare met siRNA in te spuit om ten einde vas te stel watter rol hul in die RWA-gasheer interaksies vertolk en hul noodsaaklikheid vir die oorlewing en voortplanting van die RWA uit te pluis. Die vlakke van relatiewe uitdrukking van *C002* en *14-3-3 ε* by 0h is vergelyk tussen SAM, die mees virulente RWA-biotipe, en SA1, die mins virulente RWA-biotipe in Suid-Afrika. Die resultate het gedui dat beide transkripte ‘n hoër relatiewe uitdrukking in SAM relatief tot SA1 het en dus voorspel dat *C002* en *14-3-3 ε* moontlik ‘n belangrike rol in RWA virulensie vertolk. Dit was duidelik vanuit die RT-qPCR-resultate dat suksesvolle onderdrukking van beide *C002* en *14-3-3 ε* bereik is 24h na aanvanklike blootstelling aan siRNA en dat die tydelike onderdrukking daarna afneem. Die uitdrukkingsdata wat verband hou met die inspuiting van die koringblaar-eksperimente het ongelukkig baie groot standaardafwykkings getoon, wat nie ideaal is nie. Die uitdrukking van die transkripte het dus baie tussen die individuele koringluise binne elke groep gevarieer. Dit is

waarskynlik as gevolg van die self-gemaakte koringluishokke en die metode van inspuiting van die siRNA in die koringblare wat die akkuraatheid van die resultate verhinder. Die vrugbaarheidsdata wat versamel is, was redelik onbeduidend as gevolg van die voorafgenoemde tekortkominge en dus kan ‘n akkurate en beslissende gevolgtrekking rondom die effek van *C002* en *14-3-3 ε*-onderdrukking oor die oorlewing en voortplanting van die RWA nie gemaak word nie. Beide metodes wat ingespan is vir RNAi – die kunsmatige dieet en inspuiting van koringblare benadering – het hul tekortkominge. Na oorweging van die RT-qPCR data, blyk dit dat die kunsmatige dieet metode meer akkurate en haalbare resultate produseer. Daarteenoor vestig die inspuitingmetode ‘n meer natuurlike manier van voeding vir die koringluise en gevolglik moet meer optimale hokke ontwerp en getoets word wat sal lei to meer noukeuring resultate.

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List of Abbreviations

°C – Degrees Celsius

x g – times gravity

ATCC – American Type Culture Collection

bp – base pairs

cDNA – complimentary DNA

cm – centimetre (s)

CO_2 – carbon dioxide

ddH_2O – double-distilled water

dH_2O – distilled water

DMEM – Dulbecco's Modified Eagle Medium

EDTA – Ethylenediaminetetraacetic acid

gDNA – genomic DNA

dsRNA – double-stranded RNA

FBS – fetal bovine serum

GFP – Green Fluorescent Protein

h – hour (s)

K_2HPO_4 - potassium phosphate dibasic

L27 – ribosomal protein L27

L32 - ribosomal protein L32

L:D – light to darkness ratio

N_2 – nitrogen

NaCl – sodium chloride

ng – nanogram (s)

nm – nanometre (s)

$MgCl_2.6H_2O$ - magnesium chloride hexahydrate

miRNAs - microRNAs

ml – millilitre (s)

mm – millimetre (s)

mM – millimolar

m/v – mass/volume ratio

piRNAs – piwi-interacting RNAs

qPCR – quantitative polymerase chain reaction

RISC - RNA-induced silencing complex

RNAi – RNA interference

rpm – revolutions per minute

RT-qPCR – reverse transcription quantitative polymerase chain reaction

RWA – Russian wheat aphid

SA1 – South African biotype 1

SAM – South African Mutant Biotype

SE – Standard Error

SEM – Scanning electron microscopy

Sf9 – *Spodoptera frugiperda* cell line

siRNA – small interfering RNA

SNPs – Single Nucleotide Polymorphisms

T_m – melting temperature

TAE – Tris-acetate-EDTA

µg – microgram (s)

µg/ml – microgram (s) per millilitre

µg/µl – microgram (s) per microlitre

µl – microliter (s)

µM – micromolar

USA – United States of Amerika

v/v – volume/volume ratio

Chapter 1:

Introduction

1.1 Introduction

Diuraphis noxia (Kurdjumov, Hemiptera: Aphididae), universally referred to as the Russian wheat aphid (RWA), is regarded as one of the most damaging pests of small grains across the temperate regions of the world. This phloem-feeding insect is responsible for severe systemic injury symptoms in wheat, barley, and other small grains due to the salivary proteins secreted and injected into the plant during feeding (Nicholson *et al*. 2015). It has been hypothesised that these proteins, including the enzymes present in aphid saliva, are responsible for various functions to ultimately overcome plant defences (Mutti *et al*. 2006). Obtaining knowledge of the composition of aphid saliva and its physiological functions could lead to the development of aphid tolerance or resistant plant species.

The currently available control strategies, including chemical pesticides, biological control agents, cultural control practises and RWA resistant wheat cultivars, are still very limited and rather ineffective. The process of double-stranded RNA (dsRNA)-mediated interference (RNAi) is commonly utilised to identify or validate genes encoding insecticide target proteins. The frequent application of RNAi has resulted in the development of novel methods aimed at genetically engineering plants to express dsRNA under specific conditions to act as an insect-pest control strategy pertaining to important agricultural crops (Nyadar *et al*. 2016). Studies have shown that feeding target insects with unique dsRNA results in the selective killing of species that contain the corresponding dsRNA sequence target (Hammond *et al*. 2000; Huvenne & Smagghe 2010; Wang *et al*. 2011; Noh *et al*. 2012). Therefore, host-induced gene silencing exhibits great potential to be incorporated into pest management strategies.

Four RWA salivary secretion proteins have previously been identified as potential candidate protein effectors (Cloete 2015), namely *C002*, *14-3-3 ε*, LOC100169243 uncharacterised protein, and an apolipophorins protein. The two most promising protein effectors of the above-mentioned candidates are *C002* and *14-3-3 epsilon (ε)*. *C002* is described as an aphid-specific protein that forms part of the watery saliva (Mutti *et al*. 2006, Pitino *et al*. 2011). Little is presently known about this protein at molecular level, but particular studies have shown that it is

related to aphid feeding behaviour and colonisation on plants, thus playing a key role in aphid-host plant interactions that ultimately influence survival and fecundity (Mutti *et al*. 2008; Zhang *et al*. 2015). *14-3-3 ε* is part of the 14-3-3 protein family which are responsible for modulating interactions between proteins while performing an important role in cell signalling, regulation of cell cycle progression, intracellular targeting/trafficking, cytoskeletal structure, and transcription (Aitken 2006). However, the function of the protein as a potential effector in aphid virulence is still unclear.

Therefore, in the study I aimed to establish whether the formerly mentioned potential protein effectors indeed play a key role during the interaction of the RWA and its host plant wheat. To answer this research question, several technical objectives were put forward, namely (i) to establish a cell-based system to perform *in vitro* expression and silencing of selected insect transcripts and (ii) to determine the importance of the identified proteins in the survival and reproduction of this insect pest. Thus, the first objective involves the growth and maintenance of a Sf9 cell line to be utilised for the expression and silencing of *C002* and *14-3-3 ε* and to observe the effects of RNAi on the survival of the cells. However, the Sf9 cell line contracted a presumed viral infection which led to efforts in verifying its presence through electron microscopy and PCR analysis. Attempts were also made to establish a primary cell culture using *D. noxia* embryos which could serve as a substitute cell-based system to perform the expression and silencing of the transcripts. Due to difficulties with persistent bacterial contaminations, alternative avenues had to be followed. This included the silencing of *C002* and *14-3-3 ε* via two *in vivo* methods – feeding on artificial medium containing siRNA and feeding on wheat plants injected with siRNA. Silencing was verified using RT-qPCR and the effect thereof was examined through fecundity analysis.

The layout of my thesis is as follows:

Chapter 2 of this dissertation involves a brief background on insect-plant interactions – focussing on the Russian wheat aphid (RWA) and its host plant wheat, salivary proteins acting

as elicitors in virulence, current RWA pest control strategies as well as RNAi as a future control strategy, and using cell-based protein expression systems for research.

Chapter 3 focuses on culturing the Sf9 (*Spodoptera frugiperda*) cell line, dealing with contamination, and attempts to establish a RWA primary cell culture.

Chapter 4 entails the silencing of two insect transcripts – *C002* and *14-3-3 ε* – using RNAi experiments, including feeding on artificial medium supplemented with siRNA and feeding on siRNA-injected wheat leaves, and observing its effect on the survival and reproduction of the aphids.

Chapter 5 consists of a summary of the main findings of this study and the significance thereof.

Appendix A contains data regarding the transcripts, and supplementary tables containing RT-qPCR data analysis results pertaining to chapter 4.

1.2 Preface

The findings obtained and presented in this dissertation are the outcomes of a study conducted between January 2014 and June 2016 under the supervision of Prof AM Botha-Oberholster, in the Department of Genetics at Stellenbosch University.

Research outputs:

Botha, A-M., 2016 Studying host-insect interactions using viral induced gene silencing and siRNA. International Oral Presentation, 4th International Conference on Plant Genomics, Brisbane, Australia, 14-15 July 2016.

Visser, I., and A-M. Botha, 2016 RNA interference as alternative RWA pest control strategy. Biennial International Plant Resistance to Insects (IPRI) Conference. International Oral Presentation, Stellenbosch, South Africa, 5-8 March 2016.

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Wang, Y., H. Zhang, H. Li, and X. Miao, 2011 Second generation sequencing supply an effective way to screen RNAi targets in large scale for potential application in pest insect control. PLoS One 6(4), Available: e0018644.

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Chapter 2:

Literature Review

2.1 Insects

Insects are the most diverse and abundant plant consuming species maintaining biodiversity on earth (Zheng & Dicke 2008; Sallam 2011). As insects' total numbers surpass those of any other category of animals, it is evident that they excel at adapting to different circumstances and environmental conditions. It has been documented that about 45% of the estimated 1 million insect species described, are herbivorous and depend on plants as their primary food source (Schoonhoven *et al*. 2005). Herbivorous insects have the ability to attack plants both below and above ground; therefore every plant part is at potential risk of damage brought about by these insects (Bezemer & van Dam 2005). The quantity and quality of the damage inflicted on plants vary to a great extent depending on the feeding strategy utilised by the insect (Howe & Jander 2008). The leaf-eating beetles (Coleoptera) and caterpillar (Lepidoptera) species comprise about two thirds of the recognised herbivorous insects, causing damage through their mouthparts that have specifically evolved for the action of chewing, tearing or snipping (Schoonhoven *et al*. 2005). Another category of herbivorous insects are the piercing-sucking insects that include thrips and spider mites. These herbivores utilise their tube-like structures in order to obtain the liquid content present in lacerated cells, while the soft tissue situated between epidermal cell layers is exploited by leafminers (Schoonhoven *et al*. 2005). Hemiptera insects, including aphids and whiteflies, contain specialised stylets that can be inserted in between cells to create a feeding site in the phloem (Howe & Jader 2008).

2.2 Insect pests and food production

Despite the greater bulk of insects being essential for humans and the environment, some insects exert adverse effects. According to the International Centre of Insect Physiology and Ecology (ICIPE), less than 0.5% of the total number of known insect species can be classified as pests, while even fewer of these pose a serious threat to humans (Sallam 2011). In 1947, Williams described insect pests as "any insect in the wrong place." For thousands of years crop growers have had to compete with harmful insect pests to maintain crop production for human use and consumption (Oerke 2006). Pests are able to diminish crop productivity in several

ways. In 1983, Boote *et al*. suggested that pests can be divided into different categories based on the impact they exert. The categories include: stand reducers (damping-off pathogens), leaf senescence accelerators (pathogens), photosynthetic rate reducers (fungi, viruses, bacteria), light stealers (weeds and some pathogens), accelerators (pathogens), tissue consumers (chewing animals, necrotrophic pathogens), and assimilate sappers (nematodes, pathogens, and sucking arthropods) (Boote *et al*. 1983).

Food provision has always been challenged by insect pests. The climate conditions of the tropic and sub-tropic regions around the world hold a large variety of insects due to favourable climate conditions (Sallam 2011). In these areas especially, a great need exists for strategies to successfully suppress the population densities of the various insect pest species to enable the production of adequate food supplies. This problem is exaggerated in developing countries, as the rapid annual increase in human population (about 2.5 – 3.0%) greatly exceeds the increase in food production (about 1%) (Sallam 2011). According to the International Food Policy Research Institute (IFPRI), the global demand for cereals is estimated to increase by 41% between 1993 and 2020, resulting in 2,490 million metric tons (Pinstrup-Andersen 2001). In order to meet this increased demand for cereals, improvements in crop yields are critical.

2.3 Wheat as host

Wheat (*Triticum aestivum* L.) is an important primary food staple and most consumed food crop by humans globally (Bashir *et al*. 1993; Gupta *et al*. 2008; Carter *et al*. 2012; Varshney *et al*. 2012). Bread wheat provides more than 20% of the calories intake by humans, while it is also a key source of proteins, vitamins, and minerals (Scofield *et al*. 2005; Brenchley *et al*. 2012). This cereal crop belongs to the Poaceae family and is characterised as an annual or biennial grass containing erect flower spikes and light brown grains (Taiwe 2011). As a member of the genus *Triticum*, the primary cultivated varieties of wheat include bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum durum*). Wheat is generally cultivated in spring (known as spring wheat) or in autumn (known as winter wheat), while it is harvested during late summer (Curtis 2002). After Morocco, Egypt, and Algeria, South Africa is the fourth biggest producer of wheat

on the African continent (Latham 2011). Although wheat is being cultivated across South Africa, the main production areas are located in the Free State (winter/spring wheat) and Western Cape (spring wheat) (Hatting *et al*. 2000).

With the ever increasing world population, food security is becoming an essential factor to address and maintain (Duveiller *et al*. 2007). Therefore, it is of the utmost importance for wheat grain production to increase with an annual rate of 2%, without expanding the land use past the available level (Gill *et al*. 2004). Wheat is able to grow in a diverse range of environmental conditions worldwide; therefore exposing it to a variety of pests and diseases. Oerke *et al*. (1994) reported that a 12.4% global average of actual yield loss per year occurs due to wheat diseases in both developed and developing countries. The declining wheat production in South Africa is mainly due to biotic and abiotic stresses and increasing production costs. Abiotic stresses can include acidic soils, fluctuating climate conditions, and pre-harvest sprouting following wet spells while wheat ripening is in progress (Taiwe 2011). Biotic stresses affecting wheat includes pests and pathogens. The major pathogens inflicting damage include rust disease like yellow/stripe rust (*Puccinia* Westend *f. sp. striiformis* Eriks.) and brown/leaf rust (*Puccinia triticina* Eriks.) (Taiwe 2011).

Several insects have been implicated in wheat damage worldwide. Most of the insect damage to wheat is known to be trivial or restricted to isolated regions. However, some insect pests are responsible for severe yield and forage losses (Duveiller *et al*. 2007). High population levels of feeding and chewing insects can lead to serious damages to wheat, but generally they are quite harmless. In the south eastern parts of the United States of America, the Hessian fly (*Mayetiola destructor*) acts as a serious pest of winter wheat (Flanders *et al*. 2013). It has also been reported to inflict major damage in North Africa, the Mediterranean regions, as well as regions of West Asia (Duveiller *et al*. 2007). This insect has the ability to seize tiller growth, kernel fillings or stem lodging. A correlation exists between infestations of aphids, including *Sitobion avenae* and *Rhopalosiphum padi*, and higher incidences of barley yellow dwarf virus (Duveiller *et al*. 2007). The greenbug (*Shizaphis graminum*) and Russian wheat aphid (*Diuraphis noxia*)

however, cause major devastation in several areas through the injection of a “toxin” into wheat leaves during feeding (Duveiller *et al*. 2007).

2.4 The Russian wheat aphid

Diuraphis noxia (Kurdjumov), commonly known as the Russian wheat aphid (RWA), poses as a major economic pest in various countries worldwide (Hein *et al*. 1989; Karren & Reeve 1989; Botha *et al*. 2005; Lapitan *et al*. 2007; Nicholas 2011). During 1987 – 1993, RWA infestation led to crop losses estimated at approximately $800 million in the United States alone (Morrison & Peairs 1998). As a phloem-feeding pest, the RWA predominantly affects wheat and barley (*Hordeum vulgare* L.), with the potential of causing up to 80 and 100% yield loss in these grains respectively (Nicholas 2011). Other small grains affected by the RWA, but to a limited extent, include oats, rye, sorghum, and triticale (Nicholas 2011). The RWA made its first appearance in the USA during the spring season of 1986 (Burd & Burton 1992), whereafter it was recorded as being present in all wheat-producing countries (Basky 2003) and it is now also in Australia. Also, it was reported that wheat production in the USA and South Africa is effected most severely (Basky 2003). It persisted as a severe pest in South Africa since its emergence in 1978. Field experiments indicated that RWA infestation in the summer rainfall production region of South Africa can result in crop losses of up to 90% (Du Toit & Walters 1984), while Burd & Burton (1992) reported a reduction in yields of up to 50% in susceptible varieties.

This pale green insect is characterised by an elongated, spindle-shaped body and can grow up to 1.4 – 2.6 mm in length as an adult (CABI 2013). The RWA contains a second tail-like process (supracaudal process) situated directly on top of the cauda (Summer & Godfrey 2009; Nicholas 2011). The appearance of the “double tail”, together with its shorter legs, antennae, and cornicles enables its distinction from other cereal aphids (Berner 2006). RWAs have the ability to reproduce extensively causing a rapid increase in population size, thus resulting in the swift progression of crop damage (Hein *et al*. 1989). Generally aphids’ reproducibility can be described as parthenogenic, i.e. they can reproduce either sexually or asexually. The RWA females retain their eggs inside their bodies and give birth to between four and five live

daughters carrying embryonic granddaughters per day for up to four weeks (Hein *et al*. 1989; Karren & Reeve 1989). It only takes about 7 – 10 days for the new young females to mature, therefore contributing to the rapid production of large infestations (Karren & Reeve 1989).

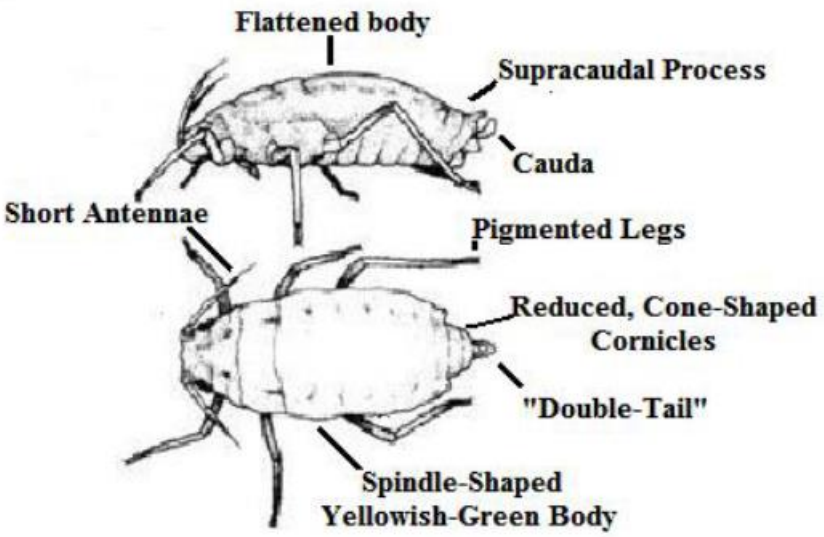

Figure 2.1: Russian wheat aphid morphology (Hein *et al*. 1989).

2.5 Plant-insect interactions

The coexistence of land plants and insects dates back more than 400 million years (Fürstenberg-Hägg *et al*. 2013). Throughout this period, plants and insects have evolved a variety of different interactions which affects organisms at all levels – from fundamental biochemical up to population genetics levels (Mithöfer & Boland 2008). Of these interactions, some are mutually beneficial like insect-mediated pollination or seed dispersion, while most are deleterious and entail insect predation of plants as well as plants conferring defence mechanisms against herbivorous insects (Mithöfer & Boland 2008; Fürstenberg-Hägg *et al*. 2013). As predator-host relationships are very common, nearly all plant species have at least one insect species preying on it (Fürstenberg-Hägg *et al*. 2013). Conventionally, insect herbivores are divided into two groups – generalists (polyphagous) and specialists (monophagous and oligophagous) (Fürstenberg-Hägg *et al*. 2013). Generalists are known to feed on hosts from diverse plant families, while specialists usually feed on one or a small number of plant types within the same family (Fürstenberg-Hägg *et al*. 2013). Insects forming

part of generalists have the ability to tolerate a wide range of defences generated in most plants, but are unable to feed on specific plants that have developed more unique methods of defence (Fürstenberg-Hägg *et al*. 2013). However, specialist insects can utilise defence compounds produced by a certain array of plants either as feeding stimulants or to provide ovipositioning cues (Fürstenberg-Hägg *et al*. 2013).

To successfully combat herbivorous attackers, plants are able to employ a diverse range of defence strategies. Some plant species are known to generate traits that influence the insect preference, including host plant selection and feeding behaviour (Fürstenberg-Hägg *et al*. 2013). Other species have the ability to affect the performance of the insects, i.e. its growth and development. The traits produced by plants can either be morphological features used as a physical defence mechanism or compounds acting as chemical defence (Fürstenberg-Hägg *et al*. 2013). Therefore plants are considered to be well defended if they are able to either escape from the herbivore preference when attacking or to reduce herbivorous performance or their population fitness post attack (Zakir 2011). Plants that can produce such traits are expected to be better represented in future generations when compared to plants that are unable to confer resistance to their attackers (Zakir 2011). Thus, even though plants lack a circulating adaptive immune system to grant it protection against insects or pathogens, they are still able to defend themselves via innate immunity (Odjakova & Hadjiivanova 2001).

The interaction between a plant and its pathogen can either be described as basic compatibility or basic incompatibility (Flor 1971). When the pathogen is able to successfully colonize its host plant and cause disease, it is described as a compatible interaction. When the attacking pathogen is unsuccessful in fulfilling this task, it is known as an incompatible interaction. Host incompatibility can result in resistance against pathogens via the activation of defence responses. Host resistance relies on the induction of a specific response due to the recognition of specific elicitors (Botha *et al*. 2005). During the interactions between plants and aphids, *R* gene products present in plants are responsible for recognising the aphid derived elicitors [avirulence (*Avr*) gene products], leading to the activation of aphid-specific resistance responses (Smith & Boyko 2007) known as “gene-for-gene” (receptor-ligand) resistance (Flor 1971). It has

been hypothesised that the *R* gene products can, either directly or indirectly, act as receptors of the *Avr* gene products [Figure 2.2 (a)] (Garcia-Brugger *et al*. 2006). When the *R*-gene products recognise the *Avr* gene products, defence related signals are produced that form part of the hypersensitive response (HR), resulting in rapid cell death (Dangl & Jones 2001; Marathe & Dinesh-Kumar 2003). The interaction between the R proteins and Avr proteins can therefore be described as the foundation of plant innate immunity, as disease will ensue in the absence of either of these proteins (Marathe & Dinesh-Kumar 2003). In spite of the above mentioned, the pathogen might want to avoid activating the defence responses and this can be achieved via pathogen *Avr* genes acquiring mutations; therefore eliciting no recognition upon entering the plant (Taiwe 2011). This results in the plant having to use alternative defence strategies. One such example is the "guard hypothesis" [Figure 2.2 (b)] during which the R protein (guard) monitors the virulence factor (non-R cellular factor), known as the "guardee." Any modifications brought about by the interaction between the Avr product and the guardee, will rapidly stimulate a defense response [Figure 2.2 (c)] (Dangl & Jones 2001; Marathe & Dinesh-Kumar 2003; Soosaar *et al*. 2005). The assumption is made that signals (avirulence effectors) are present in insect saliva, which are able to activate the incompatible interaction via mechanisms recommended in the guard hypothesis (Kaloshian & Walling 2005).

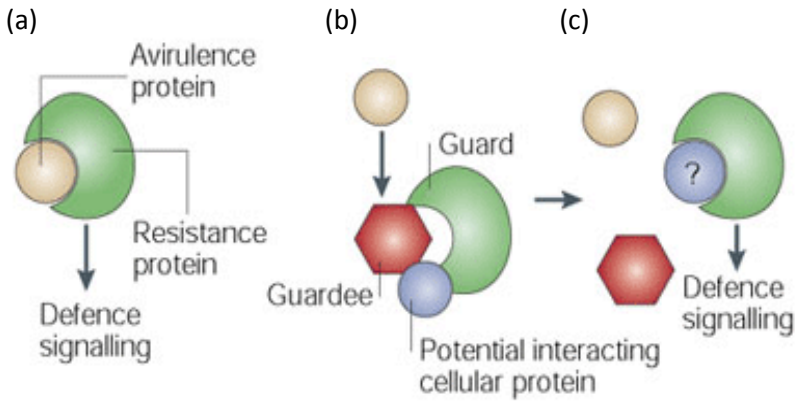

Figure 2.2: Gene-for-gene model. **(a)** The "gene-for-gene model" predicts that resistance (R) proteins detect pathogen infection by directly interacting with avirulence (Avr) proteins, leading to defence signalling. **(b)** The “guard hypothesis” entails R proteins “guarding” cellular proteins known as “guardees.” These guardees act as targets of Avr proteins and are postulated to be required for successful infection by the pathogen. A dynamic interaction exists between the guard and guardee. **(c)** Modification of the guardee by the Avr protein alters the interaction between the guard and guardee, resulting in the guard triggering a signalling cascade to enable defence (Modified from Soosaar *et al*. 2005).

2.6 The Russian wheat aphid as phloem feeding insect

Aphid feeding is thought to activate defence responses in its host plant which are similar to those triggered by viral, bacterial, or fungal pathogens (Walling 2000). The classification of RWA populations is based on the varying degree of damage they inflict on wheat plants containing different resistance (*Dn*) genes (Smith *et al*. 1992). The virulence grouping is done according to the level of foliar damage occurring due to RWA feeding (Burd *et al*. 2006). According to Puterka *et al*. (2012), host plant response can either be classified as susceptible, intermediate, or resistant. The RWA usually starts its feeding at the base of the leaves close to the upper part of the plant, where they are protected from their natural enemies (Hein *et al*. 1989). Feeding entails the probing of their stylets intercellularly until the phloem is reached (Fouché *et al*. 1984), whereafter salivary secretion is transferred leading to wide-spread damage to plant tissue (Hein *et al*. 1989; Karren & Reeve 1989; Cooper *et al*. 2011). RWA infestation

can bring about developmental, morphological, physiological, and biochemical responses in the host plant (Botha *et al*. 2005).

The phenotypic injuries to host plants (Figure 2.3) associated with RWA feeding includes chlorosis, leaf rolling, longitudinal streaking, head trapping, and stunted growth. The RWA infestation causes a continuous longitudinal white and yellow streaking to develop along the length of the leaves (chlorosis) (Hein *et al*. 1989; Nicholas 2011; Botha *et al*. 2012), while plants adopt a purplish colour (Hein *et al*. 1989; Summer & Godfrey 2009). Normally, it takes only seven days as of initial RWA infestation for these visible symptoms to manifest (Fazel-Najafabadi *et al*. 2014). RWA infestation causes a loss of photosynthetic pigments, including chlorophyll *a*, chlorophyll *b*, and carotenoids, all of which are essential for plant growth and development (Wang *et al*. 2004). In the absence of these pigments, the leaves are unable to absorb light and thus cannot store energy. Therefore the leaf chlorophyll reduction, resulting from the “toxins” injected while the aphid feeds, leads to a decrease in photosynthesis and ultimately the collapsing of the plant (Heng-Moss *et al*. 2003; Botha *et al*. 2006; Nicholas 2011). The inward rolling of the leaf edges will commence as the aphid colony develops (Hein *et al*. 1989), forming a suitable microclimate for the aphids (Hein *et al*. 1989; Nicholas 2011). This tubular structure offers the aphids protection against natural enemies and insecticidal sprays (Hein *et al*. 1989).

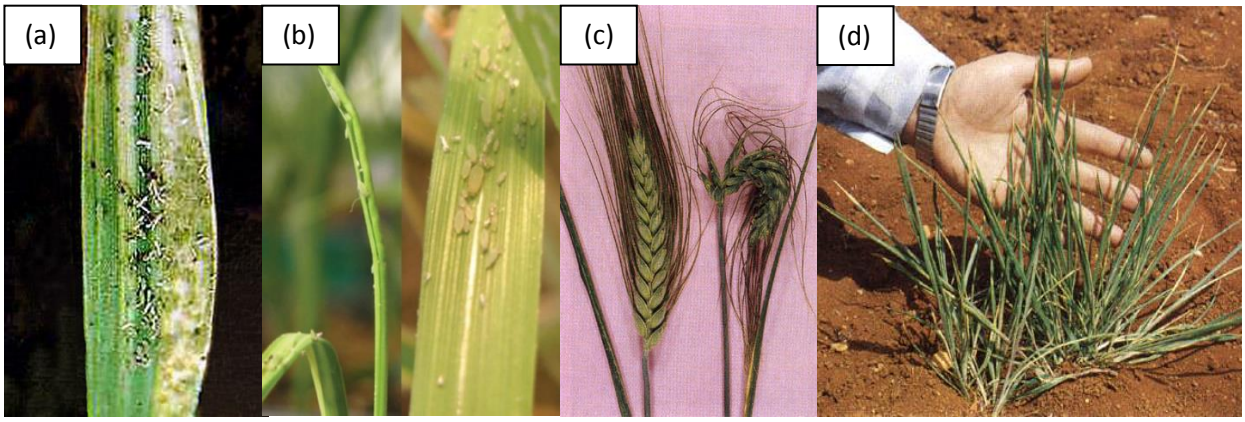

Figure 2.3: Phenotypic injuries induced by RWA infestation. **(a)** Colony of Russian wheat aphids on affected wheat leaf, indicating streak chlorotic lines (http://mazinger.sisib.uchile.cl/repositorio/lb/ciencias_agronomicas/arayaj01/p2/c11/7.html). **(b)** Leaf rolling and longitudinal streaking visible on affected wheat leaves (http://ee.oxfordjournals.org/content/43/3/672). **(c)** Head trapping resulting in 'Fish hook' deformation of a wheat head (right), due to Russian wheat aphid infestation, compared to a normal wheat head (left) (http://www.fao.org/docrep/006/y4011e/y4011e0x.htm). **(d)** Wheat plants displaying stunted growth and longitudinal streaking on tightly rolled leaves as result of Russian wheat aphid feeding (http://www.fao.org/docrep/006/y4011e/y4011e0x.htm).

2.7 RWA mouthparts and salivation

A short, triangular labrum makes up the mouthparts of the RWA and is responsible for covering the base of the stylet bundle, known as the labium (Taiwe 2011). The labium is a tubular organ that is segmented and contains complex musculature which is able to contract and shorten when the stylet is inserted into plant tissue (Uzest *et al*. 2010). Two pairs of chitinous, needle-like stylets make up the stylet bundle as seen in Figure 2.4. These pairs contain an inner pair of maxillary stylets and an outer pair of mandibular stylets, which moves independently when the leaf surface is pierced (Taiwe 2011). Interlocking grooves, situated on the inner surfaces of the maxillary stylets, fix them together. These grooves are opposed to form a food canal and a salivary canal in between (Dixon 1973). The proboscis is known as a modified labium and comprises of a sheath that holds the stylet bundle in a groove formed on its dorsal surface, as

well as five segments wherein the terminal proboscis segment grips the stylet firmly while it fixes the point of insertion (Uzest *et al*. 2010). The tip of the proboscis contains a tactile receptor that responds to the leaf surface texture, while it also enables the aphids to detect the contours of veins as this is their preferred site of feeding (Tjallingii 1978). The protraction of the mandibular is used for probing, whereafter the maxillary stylet is protracted.

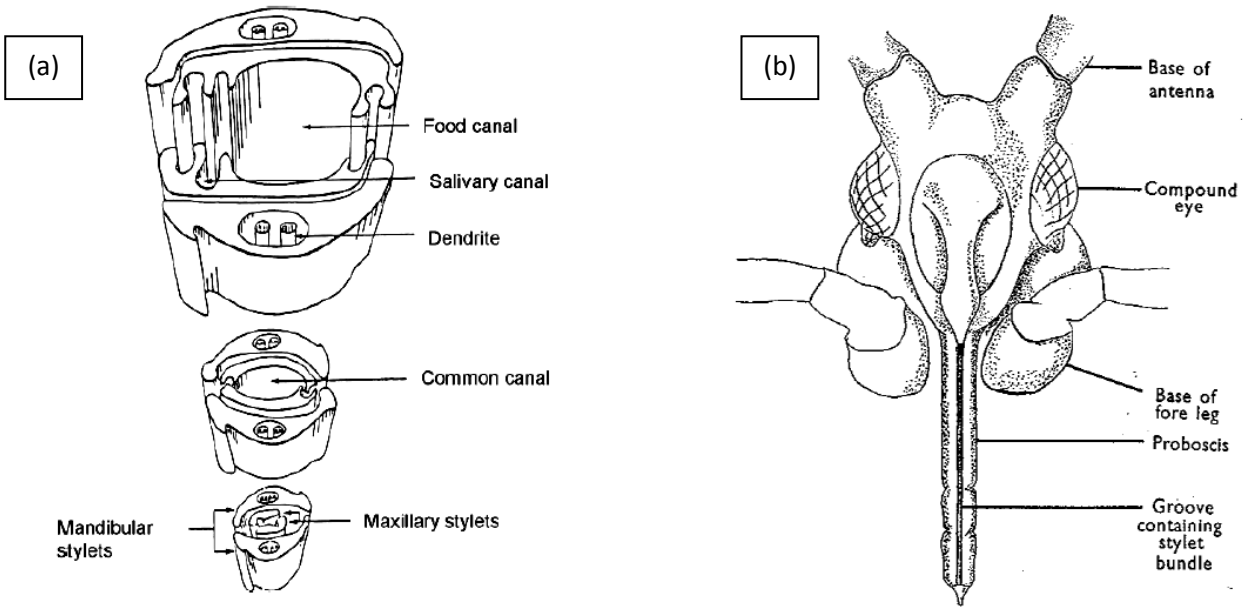

Figure 2.4: A schematic representation of the distal extremity of the stylet bundle of aphids. **(a)** Two exterior mandibular stylets surround and protect two inner maxillary stylets (Uzest *et al*. 2010). **(b)** Anterior view of the head and proboscis of an aphid to show the groove in the proboscis in which lies the stylet bundle (Dixon 1973).

Aphids possess paired salivary glands, where both the left and right gland comprises of two glandular units – a large primary gland and a smaller accessory gland (Tjallingii 2006). The primary gland is characterised by being innervated and subdivided into eight secretory cells, each possibly secreting a different component (Ponsen 1972). No innervation seems to be present at the accessory gland, and limited differentiation of its cells is observed (Tjallingii 2006). It appears as though compounds present in the saliva of aphids are able to induce defence responses in host plants. According to Miles (1972) the watery saliva of most Hemiptera has a pH between 8 and 9, making it slightly alkaline. It has been proposed that the functions of aphid saliva include: 1) food moistening; 2) to perform pre-digestion of the

compounds in the food source (hydrolytic enzymes); 3) facilitating in the "mechanical penetration" of cells while feeding commences; 4) protecting the stylet and prolonging feeding time; and 5) assisting in the dissolution of plant material as the probing events take place (Miles 1999).

The RWA releases two types of saliva into its host plant, namely gelling saliva and watery, digestive saliva. The gelling saliva is responsible for establishing a protective sheath surrounding the stylets, while the watery saliva is released into the phloem sieve elements (Miles 1999). The secretion of a small amount of gelling saliva takes place as soon as the aphid is settled on the leaf tissue (Miles 1999). A viscous blend of sheath precursors is secreted by the stylet and it gels together in the presence of air. The principal gland secretes the gelling saliva, which serves as a protective sheath surrounding the stylet throughout probing and also makes sure no sap is lost as intracellular penetration takes place (Moreno *et al*. 2011). The gelling saliva is made up of phospholipids, proteins, conjugated carbohydrates, and free amino acids (Kaloshian & Walling 2005). The watery saliva is known to contain a variety of enzymes, including: peroxidises, pectinases, cellulases, lipases, and β-glucosidases (Miles 1999). The components present in the watery saliva seem to be responsible for triggering defence signals in the plants (Taiwe 2011). It also assists with the digestion process of external and ingested food materials and the secretion of certain metabolites (Moreno *et al*. 2011). As phloem-feeding insects, RWAs secrete saliva into host plant tissue from their stylets, which are intricately narrowed to adopt a diameter of merely a few microns (Tagu *et al*. 2008). Saliva is secreted directly after the outer tissue is probed by the aphid. Leaf tissue is penetrated intercellularly via the stylets, until the phloem is reached. Watery saliva is discarded as soon as the stylet enters a phloem sieve tube, which will commence until ingestion of the plant sap is completed (Miles 1999).

Saliva plays a key role during the interaction of aphids with their host plants (Mutti *et al*. 2006). According to Miles (1999), the common assumption can be drawn that the injected aphid saliva is the damaging factor when injury occurs to food plants in the absence of virus transmission. Saliva is thought to grant aphids the ability to oppose resistance factors released by their host

plants. This notion arose from the emergence of some species, or biotypes, of aphids having the ability to feed on plants that confer resistance to other species/biotypes (Miles 1999). Therefore, the theory originated that salivary action is able to determine both the severity of aphid damage to their host plant as well as the capability of aphids to attack certain plant cultivars or species (Miles 1999). Despite aphid saliva attracting vast interest recently, still very limited information is available regarding aphid salivary components and the correlation between aphid saliva and host plant interactions (Cooper *et al*. 2010). Thus, obtaining knowledge of the composition of aphid saliva and its physiological functions could lead to the development of aphid tolerance or resistant plant species.

2.8 Proteins as eliciting agents

As previously stated, insect saliva is thought to comprise of several hydrolytic enzymes with the ability to act as elicitors (Miles 1999). If a gene-for-gene model is followed during plant-aphid interaction, the potential exists for identifying an aphid elicitor (Lapitan *et al*. 2007). Prior to the study conducted by Lapitan *et al*. in 2007, *in vitro* and *in vivo* assays proposed that elicitors are present in the RWA. Therefore, Lapitan *et al*. (2007) set out to identify RWA extracts that are able to stimulate differential responses between resistant and susceptible wheat genotypes. For this purpose they used fractionated RNA for testing. The protein and non-protein components were separated and it was found that the protein fraction is responsible for inducing the susceptibility symptoms (Lapitan *et al*. 2007). Their results indicated that when RWA extracts consisting of proteins are injected into wheat, susceptible genotypes can develop susceptibility symptoms (Lapitan *et al*. 2007). This was demonstrated by the occurrence of leaf rolling due to the injection of protein extracts that contained whole, ground aphids. Proteins were extracted from RWA biotype 2 and repeatedly injected into wheat, resulting in leaf rolling, head trapping, chlorosis, and stunted growth in susceptible plants. Whole extracts from the RWA, which contained both the protein and non-protein fraction, were unable to produce susceptibility symptoms in susceptible plants. Therefore, the results indicated that only the RWA protein extracts were able to stimulate the development of susceptibility symptoms and thus suggests that the major eliciting agents are present as soluble proteins (Lapitan *et al*. 2007). This led to

the initiation of further studies to identify the specific proteins that are responsible for causing the differential phenotypic and biochemical responses observed between susceptible and resistant wheat genotypes.

In 2010, Cooper *et al*. isolated and compared salivary proteins from RWAs feeding on three different aphid probed diets. This study set out to investigate the different aspects of salivation and the composition of RWA saliva. For the first time, protein phosphatase activity was observed in aphid saliva (Cooper *et al*. 2010). It was proposed that this enzyme is involved in the formation (also known as sclerotization) of the stylet sheath in the RWA. Cooper *et al*. (2010) also suggested that phosphatase might be implicated in aphid-host interactions such as the detoxification of host defences or host manipulation. Furthermore, a few other RWA salivary peptides displayed low but significant similarities to identified aphid ESTs (Cooper *et al*. 2010). Two of these were a putative zinc-binding dehydrogenase – an enzyme associated with alcohol detoxification – and RNA helicase. RNA helicases, which have not previously been observed in insect saliva, are known to be target-specific enzymes with the ability to modulate RNA structure (Cooper *et al*. 2010). Although Cooper *et al*. (2010) identified dehydrogenase and RNA helicase in RWA saliva, subsequent studies are necessary to confirm their presence and activity in aphid saliva as well as to identify other possible protein elicitors.

2.9 Target protein transcripts

Four RWA salivary excretion proteins have previously been identified by Cloete (2015). These proteins include *C002*, *14-3-3 ε*, LOC100169243, and LOC100159010.

In 2006, Mutti *et al*. conducted a study on the pea aphid (*Acrythosiphon pisum*) utilising *C002*, found in the salivary gland, as primary target transcript. This encoded pea aphid protein's function is completely unknown, while it includes a predicted signal peptide (Mutti *et al*. 2006). Results of this study indicated that adult parthenogenic pea aphids injected with si*C002*-RNA had a significantly reduced life-span (Mutti *et al*. 2006). Aphids injected with control si-RNA, lived well longer than the si*C002*-RNA injected pea aphids and displayed similar survival to uninjected aphids (Mutti *et al*. 2006). *C002* transcript levels were examined using RT-PCR and

Mutti *et al*. (2006) found that injected si-RNA has the ability to lower the level of this transcript. However, the ultimate function of the *C002* transcript still needs to be investigated in on-going experiments.

The second transcript is known as a 14-3-3 epsilon (ε) protein. The 14-3-3 protein family comprise of highly conserved acidic proteins with monomeric molecular weights between 28 and 33 kDa and occur in a broad range of eukaryotic cells (Tzivion *et al*. 2001; Kong *et al*. 2007; Tabunoki *et al*. 2008). To date, seven isoforms have been identified in mammals, including ε, β, γ, ζ, η, θ and δ, while thirteen are present in *Arabidopsis* and two in yeast, *Caenorhabditis elegans* and *Drosophila* (Kong *et al*. 2007; Tabunoki *et al*. 2008). In 1996, Muslin *et al*. found 14-3-3 proteins to be the first polypeptides containing phosphoserine/threonine (pSer/Thr) binding properties, thus inferring that these proteins are involved in cell signalling or signal transduction. 14-3-3 proteins' ability to form dimeric protein complexes lends them the ability to regulate protein-protein interactions (Kong *et al*. 2007). Besides signal transduction, this protein family also plays a role in the regulation of various other vital cellular processes, such as metabolism, cell cycle (timing and arrest due to DNA damage and stress), differentiation, cell development, apoptosis, transcription, protein trafficking, and malignant transformation (Kong *et al*. 2007).

The LOC100169243 uncharacterised protein identified by Cloete (2015) is unfortunately uncharacterised. The general function and the role of this transcript in providing virulence to the RWA is completely unknown.

The last transcript, LOC100159010, is described as an apolipophorins protein. Apolipophorin proteins – apolipophorin I (apoLp-I) and apolipophorin II (apoLp-II) – are the two glycosylated apolipoproteins which form the lipophorin protein (Zdybicka-Barabas & Cytryńska 2013). The apoLp-I and apoLp-II proteins, which always occur in the lipohorin particle, is joined by an exchangeable molecule, apolipophorin III (apoLp-III) (Zdybicka-Barabas & Cytryńska 2013). Together these three proteins are involved in lipid transport and the detoxification of microbial cell wall components. ApoLp-III also has a "recognition of non-self" ability which promotes insect

innate immunity by acting as a pattern recognition molecule (Zdybicka-Barabas & Cytryńska 2013). It has been proposed that all three apolipoproteins act together to achieve a coordinated defense opposing pathogens.

2.10 Traditional methods of RWA pest control

2.10.1 Chemical control

Approximately 9,000 species of insects and mites are responsible for about 14% of crop loss globally; therefore pesticide use has become crucial in agricultural production (Zhang *et al*. 2011). It is estimated that the production of nearly one-third of agricultural products rely on pesticide use (Liu *et al*. 2002), because crop losses due to pests can be reduced from 42% to 35% if pesticides are utilised (Liu & Liu 1999). South Africa accounts for 2% of pesticide use of the total 3% that Africa use (Zhang *et al*. 2011). According to the Environmental Protection Agency (EPA), pesticides can be described as any agent that is used to either kill or control undesired insects, rodents, weeds, fungi, bacteria or any other microorganism (Aspelin 1997). Therefore, insecticides, herbicides, rodenticides, fungicides, nematicides, and acaracides are collectively known as “pesticides” (Aspelin 1997).

Drought and leaf rolling by aphids restrains the use of insecticides to control RWA infestation (Du Toit 1992, Tolmay 2006). Leaf rolling, especially, necessitates the insect colonies with a protective shelter, therefore impeding the efficacy of contact insecticides (Fazel-Najafabadi *et al*. 2014; Masinde *et al*. 2014). Chemical control mainly includes foliar treatment with systemic insecticides like dimethoate (Cygon), disulfoton (Di-Syston), and demeton-s-methyl (Hein *et al*. 1989; CABI 2013), vapour action insecticides like chlorpyriphos and parathion (Nel *et al*. 2002), and seed dressings such as imidacloprid and thiametoxam (Nel *et al*. 2002). Hill *et al*. (1993) reported that chlorpyrifos caused the largest reduction in the RWA density (CABI 2013). However, the RWA displays variation in its receptiveness to insecticides suggesting that this pest has the ability to develop resistance to such chemicals (Brewer & Kaltenbach 1995).

2.10.2 Biological control

Biological control methods for the RWA have also been considered. During biological control**,** a living organism (i.e. a natural enemy of the pest) is imported and released in order to control the pest (Berner 2006). The natural enemies can include insects, viruses, fungi, bacteria, plants, and vertebrates. According to Hein *et al*. (1989), different species of lady beetles, syrphid fly larvae, and parasitic wasps can be utilised as natural control agents. These agents however, are only present in fairly low numbers and are usually not able to reduce the RWA populations beneath harmful levels (Hein *et al*. 1989). Marasas *et al*. (1997) stated that the RWA invaded South Africa without its natural enemies. Therefore, natural enemies derived from the country of origin of the pest need to be introduced in order to manage infestations. However, disadvantages of biological control include unsuccessful control of the host population before economic damage occurs, inadequate adaptation of the biological agent to the environment, and susceptibility to chemical and cultural control measures (Hajek 2004).

Aphid populations have also been reduced by employing entomopathogenic fungi displaying high epizootic potential (Taiwe 2011). These fungi include the species *Pandora neoaphidis*, *Conidiobolus obscurus* and *Entomophtora planchoniana* which are responsible for the production of microscopic spores that germinate as it comes into contact with the skin of the aphid (cuticle), whereafter it penetrates the exoskeleton and results in a fatal disease (Shah & Pell 2003). Specific species of aphids seem to be more susceptible to fungal toxins, for example *Neozygites fresenii* often attack cotton aphids, while *Therioaphids trifolli* combats spotted alfalfa aphids and *Pandora neoaphidis* is able to affect *D. noxia* (Hatting *et al*. 2000). However, entomopathogenic fungi require significant amounts of humidity in order to be effective and therefore are not likely to kill RWAs as they are predominantly present in dry regions (Taiwe 2011). Also, the toxins produced by these fungi act as slow killers and low temperatures can inhibit it.

2.10.3 Cultural control

Cultural control practices aim to establish less favourable habitats for pest populations via environmental manipulation measures (Elzinga 2000). This can either cause a disruption of the pest's life cycle or lead to improved conditions for natural enemies (van Emden 2002). Cultural control methods can be divided into three groups: prevention, avoidance, and suppression (Sotelo-Cardona 2004). Prevention and avoidance can make use of the same strategies, such as utilising pest-free seed; optimal plant nutrition, water management, and sanitation; selection of well-adapted cultivars; selection of "pest-free" planting and harvesting times; crop rotation; and trap crops (Bajwa & Kogan 2004). For example, wheat is more susceptible to damage from RWAs if they are subjected to environmental stresses (UC Integrated Pest Management Program 2016). Therefore, adequate soil growing conditions in terms of water and nutrients need to be maintained (Hein *et al*. 1989). Suppression refers to the use of crop diversification, soil tillage, optimal row spacing, and destruction of alternative hosts and volunteer-crop plants (Bajwa & Kogan 2004). For RWA management, volunteer wheat can be destroyed and removed at least two to three weeks before planting as they accumulate RWA populations and can lead to the infestation of newly planted wheat (Hein *et al*. 1989, Nicholas 2011). Also, adapting planting dates can contribute to managing RWA infestation, i.e. winter wheat can be planted as late as possible and spring grain as early as possible (Sotelo-Cardona 2004).

2.10.4 Host resistance genes and RWA biotypes

The most effective approach for RWA control presently is the use of host plant resistance genes against this pest, while it is also an environmentally and economically feasible approach (Venter & Botha 2000). In 1978, the RWA made its appearance in South Africa which was the first report of its presence outside the initial area of distribution (Jankielsohn 2014b). Initially the RWA was restricted to the Bethlehem area in the Free State Province, where infestations lead to significant wheat damage. However, by 1979 the RWA had spread and was found in other wheat producing regions of the country (Walters *et al*. 1980). At first, chemical control was used

to manage RWA infestation, but in 1990 this was replaced by the deployment of the first RWA resistant wheat cultivar, TugelaDN, containing the *Dn1* resistance gene (Marasas *et al*., 1997). In 1986, the RWA was also reported in the United States (Morrison & Peairs 1998), which resulted in the release of the *Dn4*-containing resistant wheat cultivar 'Halt' in 1994 (Quick *et al*. 1996). For years, plant resistance demonstrated effective management of the RWA. However, new RWA biotypes emerged in both countries – in 2003 and 2005 in the United States and South Africa, respectively (Haley *et al*. 2004; Tolmay *et al*. 2006). Botha *et al*. (2005) describes a new biotype as populations within an insect species that are able to damage plant entries normally conferring resistance to that insect. It was reported that the new US biotype conferred virulence to the resistant 'Halt' cultivar in Colorado. Similarly, the new biotype discovered in South Africa had the ability to overcome the resistance of the TugelaDN cultivar (Jankielsohn 2011). At present four wildtype RWA biotypes, varying in their level of virulence, are recorded in South Africa (Jankielsohn 2014b). The first biotype reported in 1978, was designated RWASA1. RWASA2 is the biotype that emerged predominantly in the Eastern Free State in 2005, displaying virulence against the *Dn1* resistance gene in wheat (Tolmay *et al*. 2007). The third biotype, RWASA3, exhibits resistance against *Dn4*-containing wheat and first appeared in 2009 (Jankielsohn 2011). This biotype is also largely present in the Eastern Free State. A new biotype resistant against the *Dn5* resistant gene was identified near Bethlehem in the Eastern Free State in 2011 (Jankielsohn 2014a). Also, a highly virulent mutant RWA biotype, SAM, has been developed (Van Zyl *et al*. 2005). SAM evolved from SA1 in the laboratory, where it was subjected to *Dn1* resistant selective pressure. The four biotypes residing in South Africa can be identified via three wheat genotypes holding the *Dn1*, *Dn4*, and *Dn5* resistance genes (Jankielsohn 2014b). According to Puterka *et al*. (2014), five main biotypes have been identified in the USA, including: RWA1, RWA2, RWA3/7, RWA6, and RWA8. These biotypes are recognised by using only four wheat genotypes that contain the *Dn3*, *Dn4*, *Dn6*, and *Dn9* resistance genes (Puterka *et al*. 2014). Puterka *et al*. (2014) stated that RWA1, RWA2, RWA6, and RWA8 biotypes from the USA differ in their level of virulence. However, the USA RWA3, RWA4, RWA5, and RWA7 biotypes were found to produce comparable virulence profiles in response to 16 cereal genotypes (Puterka *et al*. 2014). To date, a total of 14 *Dn* genes have

been identified, including: *Dn1* and *Dn2* (Du Toit 1987, 1988, 1989), *dn3* (Nkongolo *et al*. 1991a), *Dn4* (Nkongolo *et al*. 1991b), *Dn5* (Marais & Du Toit 1993), *Dn6* (Saidi & Quick 1996), *Dn7* (Marais & Du Toit 1993), *Dn8* and *Dn9* (Liu *et al*. 2001), Dnx (Harvey & Martin 1990), *Dny* (Smith *et al*. 2004), *Dn2414* (Peng *et al.* 2007), *Dn626580* (Valdez *et al*. 2012), and *DnCl2401* (Fazel-Najafabadi *et al*. 2014). After characterisation, these genes have been genetically mapped to either the 1D or 7D chromosome of wheat (Fazel-Najafabadi *et al*. 2014). The prompt emergence of various new biotypes over a short period of time has raised concerns about the durability of using plant resistance as a means of RWA control.

Limited management and control tactics pertaining to the RWA are currently available. Thus, it is evident that a significant need exists for the development of new, more effective aphid control strategies. In order to achieve this, it is of the utmost importance to acquire a better understanding of the genetic and evolutionary mechanisms playing a role during the interaction of the RWA and its host plants.

2.11 Alternative methods for RWA control

Insects and the application of insecticides lead to billions of dollars' worth of crop losses globally every year. As insecticides are continuously utilised to manage insects, it poses the risk of insects developing insecticide resistance. Over the last century, the transgenic approach has become an essential tool employed in the management of insect pests. Success has been achieved by the *Bacillus thuringiensis* (*Bt*) toxin that is utilised for the protection of various crops, also to replace chemical insecticide use to a certain degree (Katoch *et al*. 2013). Despite these successes, several vital insect pests cannot be controlled via the *Bt* protection strategy and some species are threatening to develop resistance to *Bt* (Tabashnik *et al*. 2008). No sufficient *Bt* toxin is available to exert insecticidal effects on aphids and some other phloem sap-sucking insects; therefore leading to these pests evolving from minor to major pests (Price & Gatehouse 2008; Upadhyay *et al*. 2011; Zhang *et al*. 2013). Some proteins, including chitinases, enzyme inhibitors, and lectins, have the ability to lend a degree of resistance against sap-sucking pests, but they often display low toxicity and specificity and can result in insects

undergoing physiological adaptations (Upadhyay *et al*. 2011). This highlights the importance of finding an environmentally friendly and sustainable insect pest management strategy.

2.11.1 RNAi as a future pest control strategy

Double-stranded RNA (dsRNA)-mediated interference (RNAi) is reported in plants as "post-transcriptional gene-silencing" and as "quelling" in fungi, while its application in animals is still fairly new. This process displays high specificity and therefore it has the potential of developing into a new specific method for managing agricultural pests (Huvenne & Smagghe 2010, Zhang *et al*. 2013). As it can inactivate proteins related to metabolism or reproduction, it has the potential of disabling or killing the target insects. Thus, the possibility exists that plants can be genetically engineered to express dsRNA to down-regulate vital gene functions present in pest insects, resulting in the protection of plants. Also, compared to pesticides, RNAi can be tailored to a specific pest and will therefore not kill both target and non-target species (Zhang *et al*. 2013). Several studies have found that developmental disorders or death can arise in insects after specific target genes are silenced. Therefore, by targeting these genes it can lead to the development of low toxicity and high efficiency pesticides (Zhang *et al*. 2013).

Fire et *al*. (1998) was the first to report on this process and described it as a "sequence-specific gene-silencing phenomenon triggered by double-stranded RNA (dsRNA) homologous to target mRNA, resulting in null or hypomorphic phenotypes." RNAi is a powerful tool used to silence the expression of genes of interest in a diverse range of eukaryotic organisms as well as cultured cell lines and can therefore be implemented in functional genomic studies (Agrawal *et al*. 2004; Lin *et al*. 2006; Xu *et al*. 2013; Zhang *et al*. 2013). RNAi consists of a multistep process (Figure 2.5) wherein 21-23 nucleotide active small interfering RNA (siRNA) is generated from cytoplasmic dsRNA in the cell via Dicer, an RNase III endonuclease, and incorporated into the RNA-induced silencing complex (RISC) (Tomari & Zamore 2005). RISC is a protein complex containing argonaute protein and is responsible for recognizing the complementary homologous RNA and the cleavaging/silencing thereof (Gu & Knipple 2013). Different techniques are available for introducing dsRNA into the test organism, such as microinjection, soaking or oral

feeding of an artificial diet (Yang *et al*. 2011). Small RNAs can also be grouped into three general main categories: short interfering RNAs (siRNAs), microRNAs (miRNAs), and piwi-interacting RNAs (piRNAs) (Carthew & Sontheimer 2009). When considering phylogenetic and physiological terms, siRNAs and miRNAs are thought to be the most broadly distributed (Carthew & Sontheimer 2009). These RNAs are known to yield from double-stranded precursors. The piRNAs on the other hand are predominantly present in animals, while their affect is most cleary observed in the germline (Carthew & Sontheimer 2009). Also, piRNAs appear to be derived from single-stranded precursors. Another distinct difference between these RNAs is seen in the different subsets of effector proteins they associate with. The siRNAs and miRNAs are known to bind to Argonaute proteins or members of the Ago clade, while piRNAs prefer binding to members of the Piwi clade (Carthew & Sontheimer 2009).

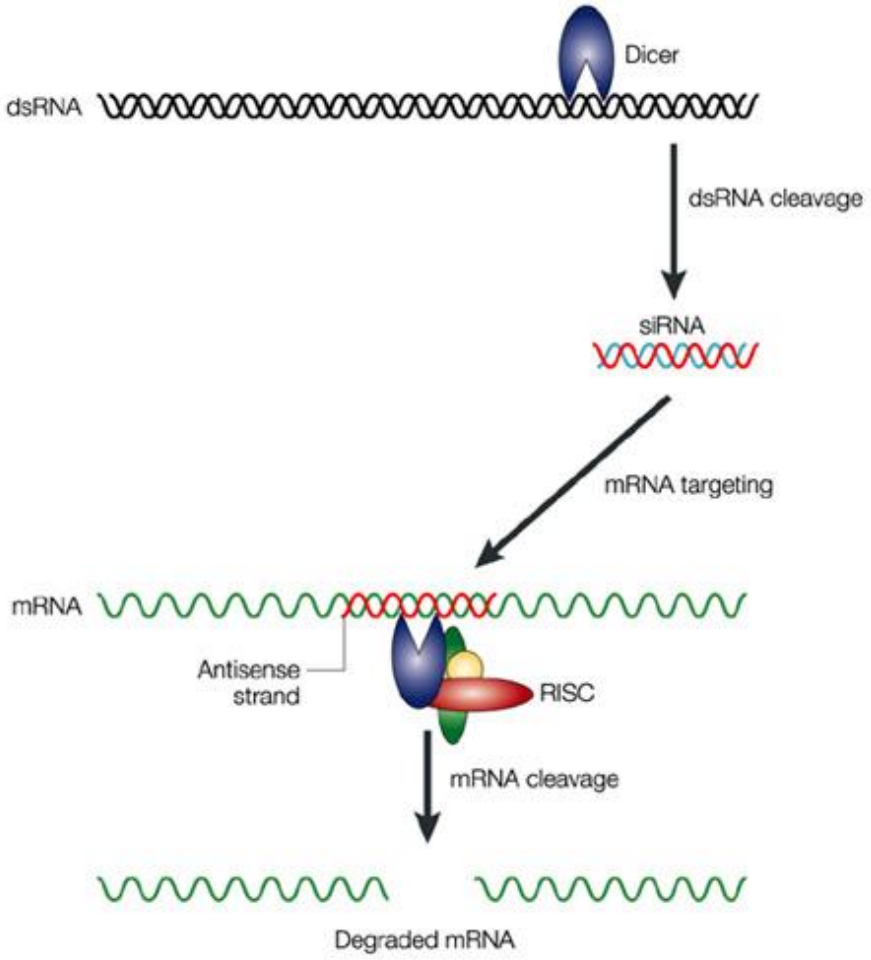

Figure 2.5: RNAi mediated gene silencing. Dicer cuts dsRNA chains into small interfering RNAs (siRNAs) of 21-23 nucleotides in length. The antisense strand of the siRNA is utilised by the induced silencing complex (RISC) to direct messenger RNA (mRNA) cleavage, leading to mRNA degradation (Modified from McManus & Sharp 2002).

Since its initial description, this technique has been employed in several entomological studies to determine regulation and expression of gene cascades. These studies were mostly conducted on *Drosophila melanogaster*, *Tribolium castaneum*, and *Bombyx mori* (Huvenne & Smagghe 2010). The first experiments performed on *D. melanogaster* followed both an *in vivo* and *in vitro* approach. For *in vivo*, embryos of *D. melanogaster* were injected (Kennerdell & Carthew 2000), while S2 cells were soaked in medium containing dsRNA as the *in vitro* route (Clemens *et al*. 2000). The first genetically transformed *D. melanogaster* lines were reported in 2000 which were created via a heritable RNAi effect that expressed dsRNA as an extended

hairpin-loop RNA (Kennerdell & Carthew 2000). These RNAi libraries have since been used for high-throughput loss-of-function studies in the dipteran model insect (Yu *et al*. 2012).

While several gene function studies have been conducted on model insects (Mutti *et al*. 2006), not a lot of work has been done on crop insects (Upadhyay *et al*. 2011). In 2011, Upadhyay *et al*. investigated the option of using RNAi to silence some essential genes in whiteflies. The dsRNA/siRNA treatments resulted in reasonable to significant mortality of the whiteflies (Upadhyay *et al*. 2011). More than 80% mortality was observed via the feeding of siRNA/dsRNA containing RPL9 and V-ATPase A subunit and higher concentrations of siRNA led to greater silencing of the target genes; therefore higher mortality (Upadhyay *et al*. 2011). Like whiteflies, aphids are also sap sucking insects. RWAs feed on phloem sap present in sieve tubes; therefore it will be critical for the dsRNA to be expressed in the phloem under the control of tissue-specific promoters (Upadhyay *et al*. 2011). Thus if the dsRNA against the vital insect genes of interest are expressed in transgenic plants, it can offer an effective mode of protection for crop plants (Price & Gatehouse 2008).

Transgenic plants have recently been reported to have the ability to produce dsRNA against target insect genes. The transgenic plant was able to confer resistance to cotton bollworm (*Helicoverpa armigera*; Lepidoptera) and Western corn rootworm (WCR; *Diabrotica virgifera virgifera* LeConte; Coleoptera), both known as economically important agricultural pests (Price & Gatehouse 2008). According to Price and Gatehouse (2008), this approach is successful due to: (i) the identification of an appropriate insect target, and (ii) dsRNA delivery of adequate amounts of intact dsRNA for uptake by the insect. Baum *et al*. (2007) identified 290 potential targets and synthesised its corresponding dsRNA *in vitro*, whereafter the effect these targets have on larval performance were determined via artificial diet feeding trials. Of the 290 potential targets, 14 genes were able to specifically down-regulate its target sequence at low dsRNA concentrations and this led to stunted growth and mortality of the insects (Baum *et al*. 2007). High larval mortality was achieved through dsRNA directed against three of the target genes - β-tubilin, V-ATPase A, and V-ATPase E – in WCR (Baum *et al*. 2007). Baum *et al*. (2007) then engineered transgenic corn able to express dsRNA targeting WCR V-ATPase A. After WCR

infestation, these plants demonstrated a significant level of protection in comparison to the controls. Thus, the transgenic plants were able to reduce the damage inflicted by WCR feeding. A second study on transgenic plants was conducted by Mao *et al*. (2007), examining the interaction between cotton and the cotton bollworm. It is believed that the suppression of the cytochrome P450 gene, *CYP6AE14*, could lead to the insect's larvae becoming more sensitive to its host plant's endogenous defense mechanism (Mao *et al*. 2007). Mao *et al*. (2007) genetically engineered *Arabidopsis* and tobacco plants to produce dsRNAs targeting *CYP6AE14* of the cotton bollworm. Plant material of both species succeeded in repressing the endogenous *CYP6AE14* transcript effectively. This repression led to the insects having an increase in its sensitivity to gossypol, a secondary metabolite of cotton (Mao *et al*. 2007).

In the present study, the RNAi silencing of genes in an insect cell line and RWA was attempted.

2.12 Cell-based protein expression systems

Generally an expression system comprises of a source of DNA together with the molecular machinery that utilises available fuels and nutrients for the transcription of DNA into mRNA, followed by the translation of mRNA into proteins. According to Biology Online (2005), an expression system can be described as a "combination of an expression vector, its cloned DNA, and the host for the vector that provide a context to allow foreign gene function in a host cell, that is, produce proteins at a high level." Increased expression quantities of the gene-encoded proteins are often observed in such systems, a phenomenon known as overexpression. Various methods are available for introducing foreign DNA to a cell in order to achieve expression. Also, a variety of host cells can be utilised namely bacteria (i.e. *E. coli*, *B. subtilis*), yeast (i.e. *S. cerevisiae*) or eukaryotic cell lines.

An attractive alternative to the frequently used lytic baculovirus expression systems, are the utilisation of non-lytic insect cell expression systems. In such a system, protein expression is achieved by transiently or stably transfecting vectors into the chromosomal DNA of insect cells (Olczak & Olczak 2006), whereafter recombinant clones are selected for and screened. Higher protein yield and more rapid expression of recombinant proteins are two of the benefits of using

a non-lytic system (Olczak & Olczak 2006). This system makes use of different cell lines which includes Sf9 and Sf21 (*Spodoptera frugiperda*), and Hi-5 (*Trichoplusia ni*).

The Sf9 cell line, clonally isolated from the ovarian tissue of the fall armyworm *Spodoptera frugiperda* (IPLB-SF21-AE), is a popular choice for research purposes (Aumiller *et al*. 2012). Sf9 is a substrain/clone of cells isolated from Sf21. Both Sf9 and Sf21 cells are able to adapt to serum-free media, while it grows well in both monolayer and suspension culture (Invitrogen 2013). These cells are suitable for experiments entailing transfection, plaque purification, generating high-titer stocks, plaque formation, as well as the expression of recombinant proteins (Invitrogen 2013). Sf9 and Sf21 cells are routinely grown in Grace's Unsupplemented Insect Medium for the expression of recombinant proteins utilising the Baculovirus Expression Vector System (BEVS) or other insect expression systems, such as the InsectSelect™ System (Invitrogen 2013). Sf21 cells are known to be a bit more unequal in size, while it forms more irregular monolayers and plaques. The High Five™ cell line (BTI-TN-5B1-4) was developed from the ovarian cells of the cabbage looper, *Trichoplusia ni* (Invitrogen 2013). This cell line is able to double in less than 24 hours, while it can grow well in adherent cultures. However, it is known to form irregular monolayers which make it more difficult to identify plaques (Invitrogen 2013). High Five™ can also adapt to suspension culture and serum-free medium and provides about 5-10 fold higher secreted proteins than that of the Sf9 cell line (Invitrogen 2013). This cell line is ideal for expressing recombinant proteins, but can also be used for transfection and plaque purification. These three insect cell lines currently dominate in academic research and commercial applications.

2.13 Establishing new insect cell lines for protein expression

The utilisation of insect cell culture as a general expression system for basic research purposes as well as large-scale commercial applications has gradually increased over the last few decades. Tremendous growth in this direction, together with more available tissue culture sources, led to the expansion of established insect cell lines (Meng *et al*. 2008; Smagghe *et al*. 2009). As research pertaining to insect cell cultures continue, new fundamental information

regarding the function of insect cells is generated. Despite the considerable growth, this field still remains at a fairly early stage of its potential development (Smagghe *et al*. 2009). Although some insect cell lines are currently being used as standard industrial tools, other established cell lines that might hold great potential have not yet been exploited. Cell culturing can be described as the removal of cells from an animal or plant, whereafter it is grown in a favourable artificial environment (Invitrogen, Cell culture basics 2015). In 1962, Dr. Thomas D. C. Grace succeeded in establishing the first insect cell lines. As a researcher with CSIRO, Grace was able to develop four cell lines from the ovaries of the female *Antherea eucalypti* moth (Lynn 1996). Currently more than 500 continuous cell lines originating from over 100 insect species are available, representing each of the economically important insect orders (Lynn 1996). These insects mostly include herbivores feeding on several host plants, causing major agricultural, forestry, and horticultural damage as pests.

Establishing new insect cell lines is crucial to enhance research pertaining to insect pathology, insect toxicology, insecticide screening, activity assays, pest control and several more (Zheng *et al*. 2014). Modern experimental biology as well as medicine, agriculture, and several biological fields have come to rely on the utilisation of insect cell culture technology (Zheng *et al*. 2014). Figure 2.6 indicates that most cell lines have been established from Lepidoptera and Diptera, while about 20% originated from all other invertebrates (Lynn 2001).

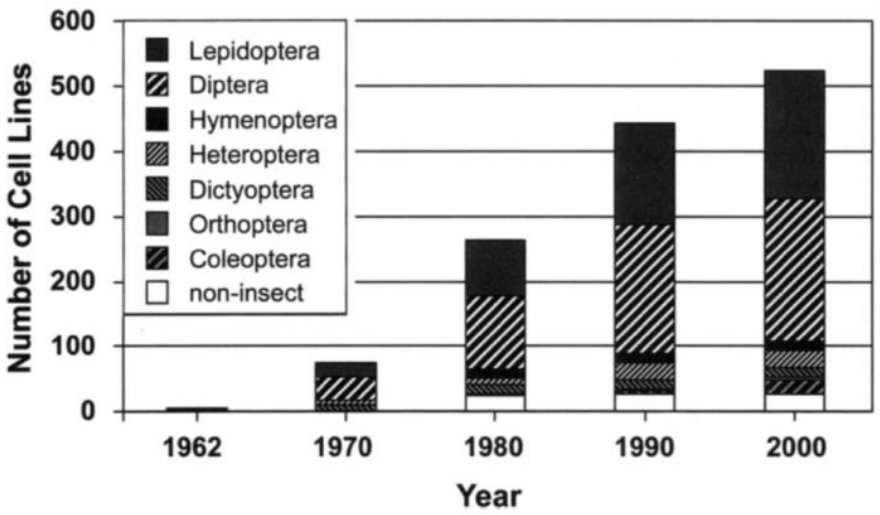

Figure 2.6: Established cell lines since 1962, originating from invertebrates and categorised by insect order (Lynn 2001).

There are two factors that render primary tissue culturing of insects a particularly difficult process. Firstly, insects are generally small and secondly, they often live in dirty environments (Lynn 1996). To overcome the relatively small size of the individuals, a colony representing a large number of insects can be used (Lynn 1996). A colony can also be cared for in such a way that microbial contamination is largely minimised (Lynn 1996). Another solution is to set up the primary cultures in small volumes and to employ antibiotics. Although it is not usually advised to use antibiotics in continuous cell lines, they can be rather valuable during the initiation of new cell lines (Lynn 1996). However, the most important factor to consider when attempting to develop a new cell line is the medium. Several factors play a role when choosing an appropriate medium, such as the pH, osmolarity, and the amount and ratio of inorganic salts (Lynn 1996). Commercial media are currently available. For Lepidoptera, “Grace’s medium” seems to be the golden standard to use, while there are also highly defined serum-free media available such as ExCell 401 (JRH Biosciences), Sf900 (GIBCO), and Insect-Xpress (Lynn 1996). On the other hand, Schneider’s *Drosophila* medium (GIBCO) and Shield and Sang’s M3 medium for mosquito cell cultures (Sigma) can be used for Diptera cell lines (Lynn 1996).

Figure 2.7 indicates all the tissues that have successfully been used for the development of insect cell cultures, which include ovaries, embryos, hemocytes, imaginal discs, fat body, midgut, neonate, and cuticle/nervous system/endocrine system/muscular system (Lynn 2001).

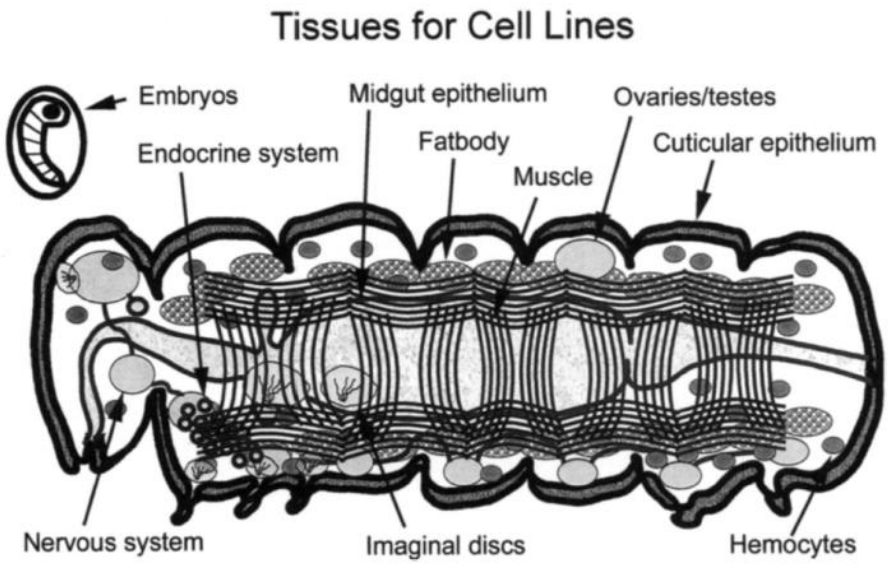

Figure 2.7: Diagrammatic representation of an insect indicating the sources of tissues that can be utilised for cell line development (Lynn 2001).

Ovaries were generally employed during the 1960s and 1970s and were mostly used to establish cell lines originating from Lepidoptera (Lynn 2001). Hemocytes are known to be easily obtained, however it takes more effort to grow. Imaginal discs are crucial for gaining knowledge about developmental biology as this is developmentally determined to give rise to specific structures while it consists of undifferentiated cells (Lynn 2001). Fat body is sometimes used as target tissue for several insect pathogens. It expresses various functions that are similar to the mammalian liver and is therefore an important source of physiological tissue (Lynn 2001). Tissue collected from the midgut is mostly used for pest control and pathological studies. Despite the fact that a couple of cell lines originating from fat body or the midgut are available, cell lines from these tissues are not always achievable as terminal differentiation and others factors play a role (Lynn 1996). Neonates contain a large amount of undifferentiated cells despite being more developed than cells found in embryos (Lynn 2001). The cuticle/nervous system/endocrine system/muscular system is mostly employed for studies pertaining to pest control, physiology, and pathology but are generally rare and non-existent cell lines (Lynn 2001). About half of the cell lines available were established from embryos, making this the most frequently used cells for culturing. Embryos are a useful source of cells, especially if a

colony of the insect is available, because large quantities can be obtained and the insect chorion is appropriately resistant to simple disinfectants which are routinely used for decontaminating the eggs (Lynn 1996). According to Lynn (1996), embryos seem to be the most useful source of cells for establishing a new cell line.

Several insect cell lines have been established from embryonic tissue. One such example is the new cell lines that were developed from the embryos of *Ephestia kuehniella* by Lynn and Ferkovich (2004). *Ephestia kuehniella*, a Mediterranean flour moth, is known as a severe pest of stored food products, mostly whole and milled grains (Lynn & Ferkovich 2004). Lynn and Ferkovich (2004) used two to four day old eggs to initiate primary cell cultures. Only one of the eight initial primary cultures displayed sufficient growth and could be sub-cultured, resulting in the first reported continuous cell line for this insect. Recently three new insect cell lines were established from the embryonic tissue of *Holotrichia oblita* Faldermann (Coleoptera: Scarabaeidae) (Zheng *et al*. 2014). *Holotrichia oblita* is an important soil pest present in Northern China and cell lines were developed to provide tools for physiological studies and to use for *in vitro* bioassays and insecticide screening studies (Zheng *et al*. 2014). Zheng *et al*. (2014) was successful in establishing QAU-Ho-E-3, QAU-Ho-E-4, and QAU-Ho-E-6 and to date these cell lines have been passaged more than 50 times, with all three displaying adherent growth.

Since the first cell line was established, the media, culture methodology and conditions have been well resolved to enable the routine development of new cell lines. However, the culturing of a specific cell type can be a difficult task. In order to assist with the future growth and application of insect cell lines, it is essential to continue developing new cell lines. In the present study an attempt was made to develop the first RWA cell line.

To conclude, in the present study the main aim was to establish whether selected insect proteins identified as potential candidate effectors play an important role during the RWA-wheat interaction and thus to form part of the basis of studies pertaining to the development of a RNAi based method of insect pest control possibly using these transcripts as targets.

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Chapter 3:

Exploring cell-based protein expression systems for *in vitro* analyses of selected insect transcripts

3.1 Introduction

Over the past 20 years, eukaryotic cells have been utilised more readily for the expression of recombinant proteins (Unger & Peleg 2012). In the case of heterologous protein expression, using an insect cell culture is a popular option. Insect cells have the ability to express great quantities of protein with complex post-translational modifications in a fairly short time-period, therefore making it applicable for both large scale production and basic research. Nowadays, insect cell cultures are widely employed for studies pertaining to insect physiology, pathology, and developmental-, microbial- and molecular biology (Lynn 2002).

Cell culturing broadly refers to the laboratory growth of cells derived from animals or plants. In its simplest form, cell culturing comprises of the distribution of cells in an artificial environment compiled of nutrient solution, an appropriate surface for cell growth support, and favourable temperature, humidity, and gaseous atmosphere conditions (Coriell Institute 2015). Since the early 1900s, cell culturing practises have been utilised for medical research pertaining to tissue growth and development, properties of cancer cells, virus biology, aging studies, genetics, and gene therapy (Hay *et al*. 1994). As of late, the field of biotechnology has also expanded into using large-scale cell culture systems for the production of great volumes of biopharmaceuticals. Cell culturing is advantageous in its simplicity compared to studies utilising whole plant or animal organs which contain a variety of cell types. The most prominent limitations of cell culturing include unforeseen contamination with microorganisms or viruses and potential cross-contamination with other cell types.

Preparing primary cell cultures are quite laborious and can usually only be maintained *in vitro* for a limited amount of time, while primary cells display several of the differentiated characteristics of the cell *in vivo* (Sigma-Aldrich 2010). As soon as the primary culture vessel has grown and filled up all of the available culture substrate, sub-culturing has to be performed in order to provide more space for continued growth. Sub-culturing entails the inoculation of cultured cells into fresh culture vessels. The product of the first sub-culture of a

primary culture is referred to as a secondary culture or cell line. Cell lines comprising of particular cell types can be established via cloning, selective culture or physical cell separation (Shenoy 2007). These cell lines are grown and maintained within a cell incubator under suitable conditions. Regular cells normally undergo a limited number of divisions before losing their ability to proliferate, thus senescence occurs, and are referred to as finite cell lines (ThermoFisher Scientific 2015). Many cell lines developed from animal tissues are finite, but others can be continuous cell lines. Continuous cultures consist of only one cell type (Sigma-Aldrich 2010) and have undergone a genetic transformation to enable an infinite growth potential. Despite cells originating from cell cultures having a limited lifespan, some cells can sporadically continue multiplying due to transformation and are called an immortal cell line, because it can be cultured indefinitely (Shenoy 2007). A cell strain is known as a subpopulation of a cell line that has been positively selected from the culture via cloning or another method. Often, a cell strain will have acquired additional genetic changes since it was established from the parent cell line.

More than 500 insect cell lines have been developed from over 100 insect species, including the frequently utilised lines originating from *Drosophila melanogaster* (fruit fly), *Trichoplusia ni* (cabbage looper), and *Spodoptera frugiperda* (fall army worm). *S. frugiperda* (Figure 3.1) is a polyphagous lepidopteran pest greatly affecting agricultural practices as it can feed on more than 40 plant families (Giraudo *et al*. 2015). In 1977, Vaughn *et al*. successfully isolated the *Spodoptera frugiperda* cell line IPLB-Sf21, commonly known as Sf21. Sf21 was derived from a pupal ovary tissue culture in Maryland, USA, at the USDA Insect Pathology Laboratory (IPLB) (Lindskog 2006). Sf9 is a clonal isolate of the primary culture IPLB-SF21 AE and is possibly the most frequently used insect cell line nowadays. Although Sf9 and Sf21 are similar in characteristics, Sf21 is known to possess a broader size distribution and more irregular monolayers and plaque formation in comparison to Sf9 (Hink *et al*. 1991). The Sf9 cell line is very susceptible to infection with Autographa California nuclear polyhedrosis virus (AcNPV baculovirus), and compatible with all baculovirus expression vectors. This cell

line is regularly utilised for the isolation and propagation of recombinant baculoviral stocks as well as the production of recombinant proteins.

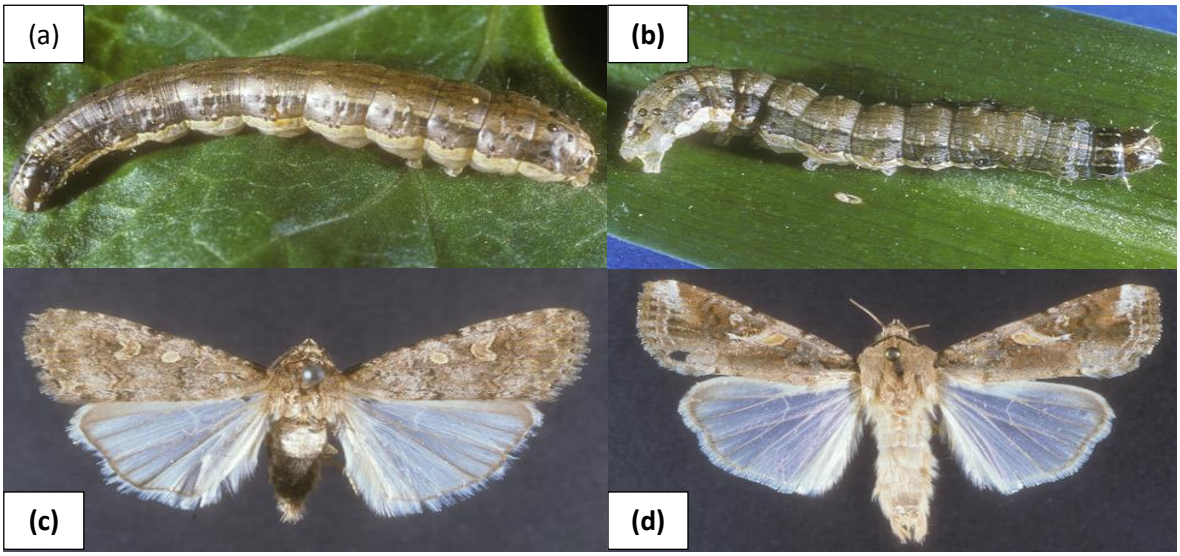

Figure 3.1: **(a)** and **(b)** Larva of fall armyworm, *Spodoptera frugiperda*. Typical adult female **(c)** and male **(d)** fall armyworm, *Spodoptera frugiperda*. Photographs by John L. Capinera, University of Florida (http://entnemdept.ufl.edu/creatures/field/fall_armyworm.htm).

Although the initial aim of the present study was to utilise the Sf9 cell line to express and silence specific protein transcripts to investigate their sites of expression and effects on survival and reproduction, unforeseen viral contamination called for a detailed investigation/detection of the specifics regarding such a contamination, before proceeding. In addition, the development of a primary cell culture taken from *D. noxia* (RWA) embryos was initiated with the eventual aim to use this cell-line for silencing of the transcripts.

3.2 Materials and methods

3.2.1 Sf9 cell culturing

The Sf9 cell line (CRL-1711, Lot no. 62058094) (ATCC®, USA), clonally isolated from the ovarian tissue of the fall armyworm *Spodoptera frugiperda* (IPLB-SF21-AE), was routinely cultured and maintained as an adherent culture in Grace's Insect Medium (1X)

Supplemented (Gibco®, Life Technologies™, USA) containing lactalbumin hydrolysate and yeastolate not commonly found in the unsupplemented version. The growth medium was supplemented with 10% v/v fetal bovine serum (HyClone™, USA) and Gentamycin/Amphotericin B (10 µg/ml gentamicin; 0.25 µg/ml amphotericin B) (Gibco®, Life Technologies™, USA). Insect cells were maintained in a non-humidified incubator at 27°C with no CO_2 exchange. The cell density and viability was determined through cell counting using the TC20™ Automated Cell Counter from Bio-Rad after dilution of the culture bulk samples in 0.4% trypan blue. The culture flasks were observed with the bare eye as well as examined with an Olympus IX51 Inverted Microscope on a daily basis.

3.2.2 Investigating Sf9 cell line for fungal/viral contamination

3.2.2.1 Screening for fungal contaminant

DMEM growth medium (Gibco®, Life Technologies™, USA) containing phenol red was obtained from a colleague and was used to observe whether a fungal contaminant was present within the Sf9 cells. DMEM (Dulbecco's Modified Eagle Medum) is a widely used basal medium for supporting the growth of many different mammalian cells and contains a four-fold higher concentration of amino acids and vitamins than the original Eagle's Minimal Essential Medium. A volume of 3 ml of cell culture was added to 15 ml of aforementioned supplemented growth medium and was incubated at 37°C and observed for colour change of the medium.

3.2.2.2 Electron microscopy sample preparation and imaging

Vials of frozen Sf9 cells were maintained in liquid nitrogen vapour phase. One vial was thawed and prepared for scanning electron microscopy (SEM) imaging at the Central Analytical Facilities (CAF) Imaging Unit in Stellenbosch (Goldsmith & Miller 2009). After centrifugation at 200 x *g* for 10 minutes, the pellet was covered with 2.5% gluteraldehyde and incubated in the fridge overnight. Cells were centrifuged to remove the gluteraldehyde

and resuspended in 0.1 M phosphate buffer (pH 7.4). The phosphate buffer was replaced with osmium tetroxide [10 ml ampule 4% osmium tetroxide; 8 ml Palade's buffer (2.89 g sodium barbitone; 1.15g sodium acetate; 100 ml dH_2O); 8 ml 0.1 M HCl; 4 ml dH_2O] and incubated at room temperature for 1 hour, whereafter the cells were rinsed with dH_2O and centrifuged for 4 minutes. A series of ethanol incubation steps was then performed, each followed by a dH_2O washing step and centrifugation for 4 minutes to pellet the cells and carefully remove supernatant. The incubation steps were as follow: 70% (v/v) ethanol for 5 min (X2); 96% (v/v) ethanol for 5 minutes; 100% (v/v) ethanol for 10 min (repeated X2 – first for 15 minutes and then for 20 minutes); 100% ethanol mixed with resin (1:1) for 90 minutes, and lastly pure resin for 1 hour (X2). The sample was then embedded in a clean dry capsule and left to dry in oven at 60˚C overnight.

The final SEM sample preparation was performed by CAF. Visualisation of samples was conducted using a Zeiss MERLIN FEG® Scanning Electron Microscope with a GEMINI II® column.

3.2.2.3 Polymerase chain reaction (PCR) analysis

3.2.2.3.1 RNA, cDNA and gDNA extraction from Sf9 cells

Frozen Sf9 cells were used for total RNA extraction with In-Column DNase I digestion, according to the manufacturer's instructions using the Direct-zol™ RNA MiniPrep Kit (Zymo Research, USA). The purity and integrity of the isolated RNA was assessed through 1.5% (m/v) agarose gel electrophoresis run in Tris-acetate-EDTA (TAE) buffer [40 mM Tris, 20 mM Acetic acid and 1 mM EDTA (Ethylenediaminetetraacetic acid)], pH 8, using ethidium bromide staining (2.5 μg/ml) and absorbance at 260 and 280 nm in a NanoDrop Spectrophotometer. The iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA) was used for cDNA synthesis, while DNAzol® Reagent was utilised to isolate genomic DNA (gDNA) from frozen Sf9 cells. RNA, cDNA and gDNA were isolated from the Sf9 cells, as the suspected contaminant was thought to be either a RNA or DNA virus.

3.2.2.3.2 PCR and gel electrophoresis

PCR was carried out using the extracted Sf9 gDNA and synthesised cDNA as template for the primers (Table 3.1) designed to specifically amplify DNA sequences pertaining to the coding regions of three highly conserved genes of Lepidopteran-specific baculoviruses, namely polyhedron/granulin (*polh/gran*), late expression factor 8 (*lef-8*), and late expression factor 9 (*lef-9*) (Lange *et al*. 2004) (Table 3.1). To determine if amplification of the correct sequences were achieved, 1.5% (m/v) agarose gel electrophoresis (TAE buffer, pH 8) was conducted using ethidium bromide staining.

Table 3.1: Degenerate oligonucleotide primer sequences for PCR using Sf9 synthesised cDNA and extracted Sf9 gDNA.

Target gene	Primer name	AcMNPV genome position	Tm (°C)	Sequence[a,b]
Polh/gran	prPH-1	42075 – 42088	38 – 54	TGTAAAACGACGGCCAGTNRCNGARGAYCCNTT
	prPH-2	41373 – 41389	38 – 52	CAGGAAACAGCTATGACCDGGNGCRAAYTCYTT
Lef-8	prL8-1	49748 – 49763	38 – 47	CAGGAAACAGCTATGACCCAYGGHGARATGAC
	prL8-2	50027 – 50043	50 – 60	CAGGAAACAGCTATGACCAYRTAS$_1$GGRTCYTCSGC
Lef-9	prL9-1	4684 – 4698	40 – 53	CAGGAAACAGCTATGACCAARAAYGGTAYGCBG
	prL9-2	5210 – 5224	47 – 57	TGTAAAACGACGGCCAGTTTGTCDCCRTCRCARTC

[a](B = C, G, or T; D = A, G, or T; H = A, C, or T; I = Inosin; N = C, A, T, or G; R = A or G; S = C or G; Y = C or T).

[b]Underlined nucleotides indicate standard sequencing primers (-21) M13 forward and (-29) M13 reverse.

3.2.3 Culturing of RWA primary cell culture and establishment of cell line

3.2.3.1 Isolating RWA nymphs

SAM aphids were collected from the Cereal Genomics Laboratory at the Department of Genetics, Stellenbosch University. The adult parthenogenic RWA females were surface-sterilised by immersion in 70% (v/v) ethanol for at least 5 minute and then placed on a sterilised slide. After the ethanol dipping, the insects were dissected in Ringer-Tyrode solution (0.8% m/v NaCl; 0.02% m/v $CaCl_2 \cdot 2H_2O$; 0.02% m/v NaH_2PO_4; 0.02% m/v KCl; 0.01% m/v $MgCl_2 \cdot 6H_2O$; 0.012% m/v $NaHCO_3$; 0.8% m/v glucose), which is a balanced salt solution modified from Locke's solution (Tokumitsu & Maramorsch 1966). The embryos were removed under a microscope (Labotec, SA) using specialised needles (Ted Pella Inc., USA) (Figure 3.2). The aphid was kept stable by placing one microdissection needle on the head, while a second needle was used to make an incision at the tail. The nymphs were carefully isolated by applying pressure on the abdomen of the adult aphid. Approximately 50 embryos were isolated (normally 3-6 nymphs per adult) and placed in a 2 ml Eppendorf tube containing Ringer-Tyrode solution. Centrifugation for 5 minutes at 13 000 rpm were performed and the pellet was re-suspended in 70% (v/v) ethanol and incubated for 5 minutes (García *et al*. 1995). Embryos were pelleted again by centrifugation at 13 000 rpm for 5 minutes. The cells were washed three times with sterile dH_2O with a centrifugation step at 13 000 rpm for 3 minutes each, whereafter the pellet was re-suspended in Grace's Insect Medium (1X) Supplemented (Gibco®, Life Technologies™, USA) containing 10% v/v Fetal Bovine Serum (FBS) (HyClone™, USA), Penicillin-Streptomycin (10,000 Units/ml penicillin; 10,000 µg/ml streptomycin) (Gibco®, Life Technologies™, USA) and Gentamicin/Amphotericin B (10 µg/ml gentamicin; 0.25 µg/ml amphotericin B) (Gibco®, Life Technologies™, USA) and the embryos were crushed using a micro-pestle. The crushed embryos were transferred to a T25 culture flask and incubated at 27°C in a non-humidified incubator without CO_2.

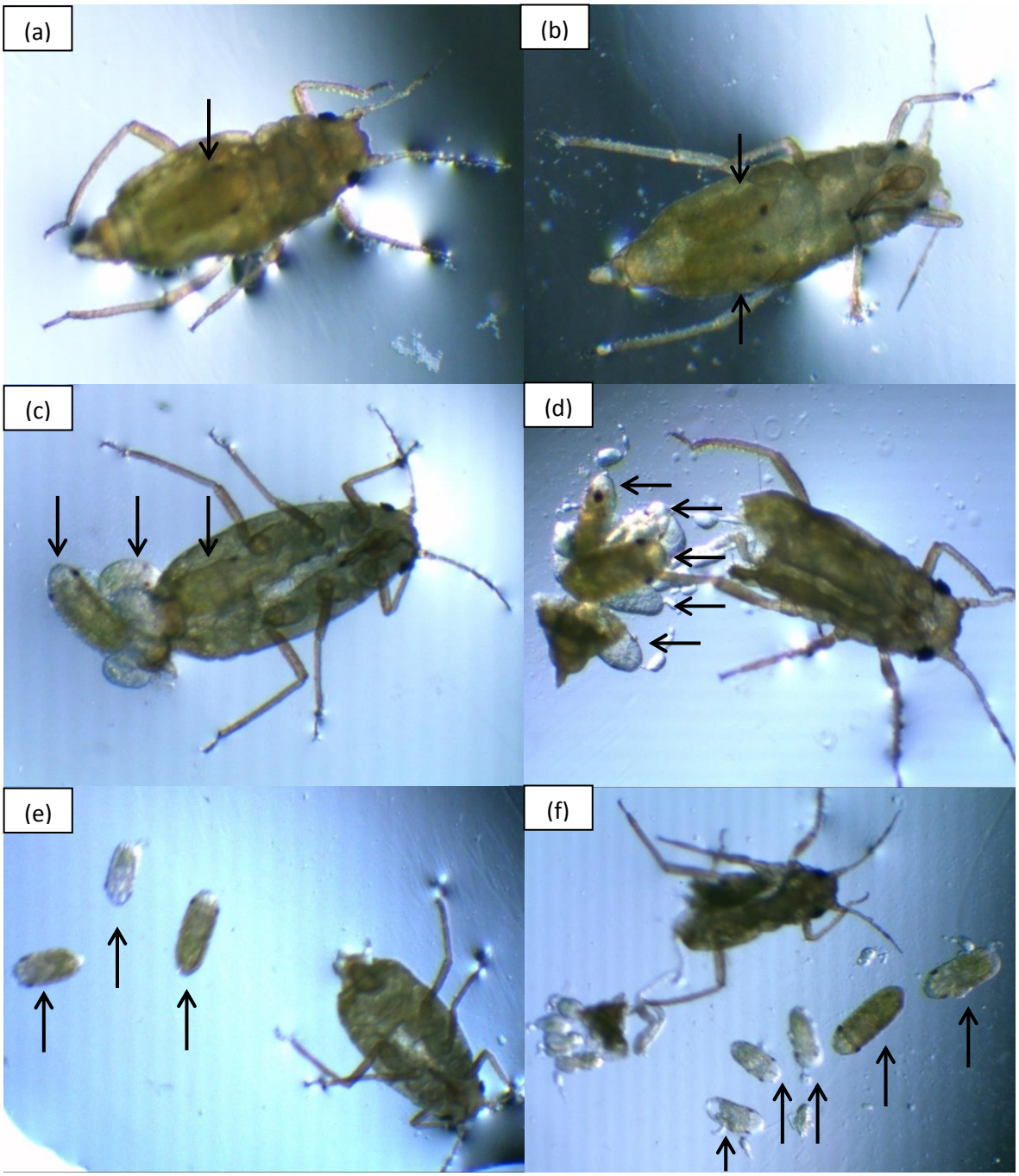

Figure 3.2: Isolating SAM nymphs. **(a)**, **(b)** Adult parthenogenic female SAM aphids in Ringer Tyrode Solution on a microscope slide. Nymphs are visible in the lower abdomen of the adult aphids (as indicated by the arrows). **(c)**, **(d)** A small incision was made at the tail of the adult aphid using specialised needles and the nymphs (indicated by arrows) were carefully released from the abdomen by applying a light pressure. **(e)**, **(f)** Usually 3-6 nymphs were isolated from each adult aphid. Photos were taken with a DCM510 5M pixel digital camera attached to the microscope.

3.2.3.2 Maintaining the primary RWA cell culture

Culture flasks were observed under an inverted microscope daily, to monitor cell growth and indications of contamination. Medium was replaced with fresh medium every 1-2 days, depending on observations. In the case of milky/white culture medium being observed, the medium was removed and centrifuged at 1000 rpm for 5 minutes. The pellet was re-suspended in fresh medium with an additional dose of penicillin-streptomycin (2X). The culture flask was carefully rinsed with sterile Ringer-Tyrode solution before the fresh medium was added.

3.3 Results

3.3.1 Sf9 adherent cell culture

The initial viability of the Sf9 cell culture was determined at 80% during the cell count performed on day 2 (24h after initiation of cell culture from frozen stock) (Table 3.2). No signs of contamination were observed and the cells were attaching to the bottom surface of the culture flask while several floating cells were visible. The latter was due to the cells adapting to new medium and coming out of its quiescent state. On day 4, the culture appeared to have reached confluency as the bottom of the culture flask was ~90% covered in cells [monolayer cultures should be sub-cultured at ~90% confluency when cells are in mid-log phase of growth (Invitrogen 2013)] and a cell count was performed. The culture, initially in a T25 culture flask (Figure 3.3), was passaged and seeded into two new T25 flasks.

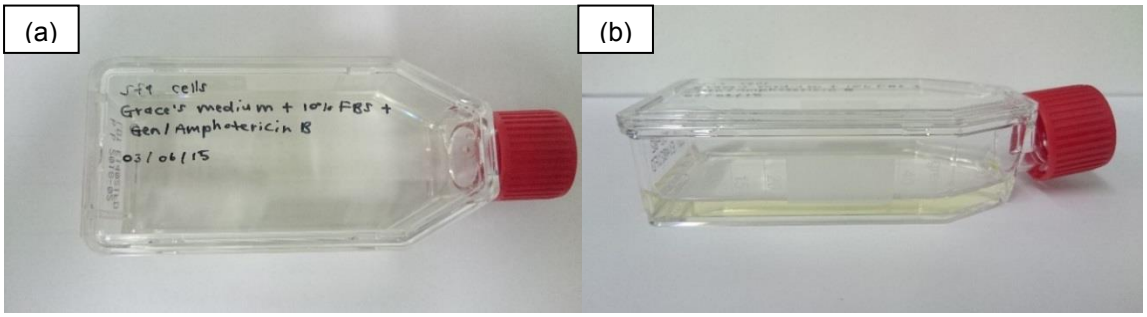

Figure 3.3: Sf9 cell culture flask. **(a)**, **(b)** T25 culture flask containing Sf9 cells in Grace's Insect Medium supplemented with 10% fetal bovine serum and Gentamycin/Amphotericin B.

Upon investigation under the microscope on day 5, the cells appeared to be floating in the medium more than attaching to the bottom surface. However, no signs of contamination were visible and the cell count indicated viability of 76% and 74% (Table 3.2) for flask 1 and 2, respectively. On the morning of day 6, a high density of cells were observed in the centre of the flasks (almost appearing confluent) while the surrounding areas contained a lower density of cells (not ~90% confluent). Later that evening, the cells in each of the flasks seemed to have reached ~90% confluency. Therefore a cell count was performed (Table 3.2) and both flasks were split and seeded into two new T25 flasks each. Thus, a total of four T25 flasks with Sf9 cells were incubated.

Table 3.2: Sf9 cell counts performed. On day 5 and 6, the first column represents flask 1 and 2, respectively.

	Day 2	Day 4	Day 5		Day 6	
			Flask 1	Flask 2	Flask 1	Flask 2
Total (cells/ml)	5.9×10^5	2.8×10^5	2.5×10^5	2.1×10^5	2.4×10^5	2.8×10^5
Live (cells/ml)	4.7×10^5	2.2×10^5	1.9×10^5	1.6×10^5	1.8×10^5	2.4×10^5
Dead (cells/ml)	1.2×10^5	6.0×10^4	6.0×10^4	6.0×10^4	7.0×10^4	4.0×10^4
Viability	80%	79%	76%	74%	73%	85%

No signs of contamination were observed on day 7 and 8, however on the latter the cells appeared smaller in size and quite dispersed in their distribution. Generally Sf9 cells are spherical with some granular appearance, but on day 9 and 10 the cells appeared swollen with some small circular objects in between them. Due to the size and appearance, it was initially thought to be yeast. Therefore, the cultures were treated with an additional 2X anti-fungal dosage of Gentamycin/Amphotericin B (10 µg/ml gentamicin; 0.25 µg/ml amphotericin B) (Gibco®, Life Technologies™, USA). However, the cells continued to swell and lyse despite persisting with anti-fungal treatment.

3.3.2 Screening infected Sf9 cells with phenol red

To test whether the apparent contamination was of fungal origin, some of the cell culture was added to a fresh DMEM growth medium containing phenol red as a pH indicator and incubated at 37˚C as this is the optimal growth temperature for yeast. However, the colour of the medium did not change and thus the presence of a fungal infection in the Sf9 culture could not be verified (Figure 3.4).

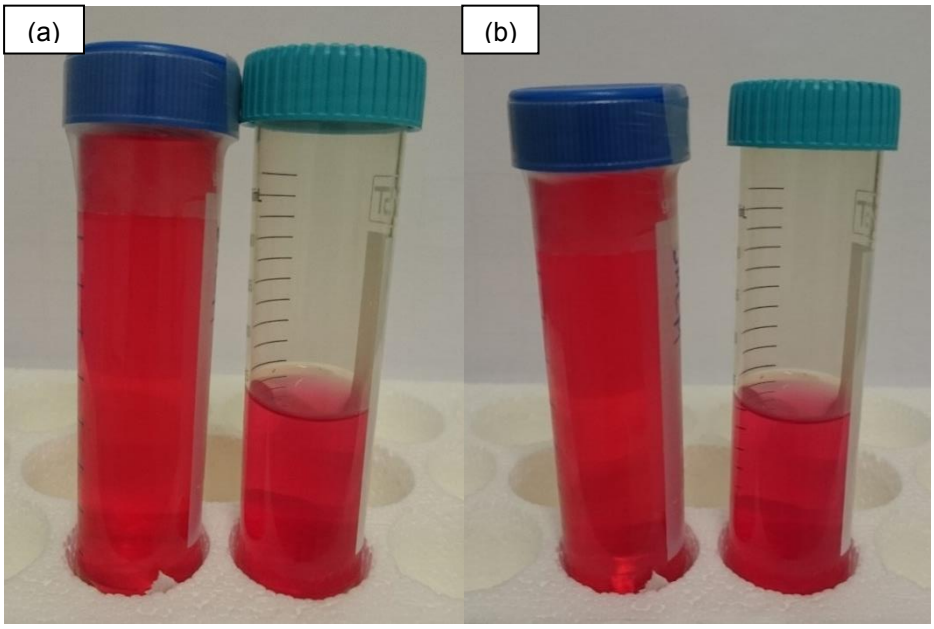

Figure 3.4: Screening for fungal contamination in Sf9 cell culture. **(a)** Control falcon tube containing only DMEM growth medium supplemented with phenol red (left) and falcon tube with DMEM with phenol red containing infected Sf9 cell culture, before incubation at 37˚C. **(b)** Control tube and tube containing Sf9 cell culture, 3 days after incubation at 37˚C. No change in the colour of the medium containing the Sf9 cells is observed.

3.3.3 Scanning electron microscopy

Baculovirus virions are characterised by a complex structure containing an envelope and rod-shaped nucleocapsid (200-450 nm in length and 30-100 nm in diameter). The CAF Imaging Unit at Stellenbosch University performed SEM analysis (60,000 X magnification) of the prepared Sf9 samples. Unfortunately, no viral particles could be observed in the Sf9 cells (Figure 3.5).

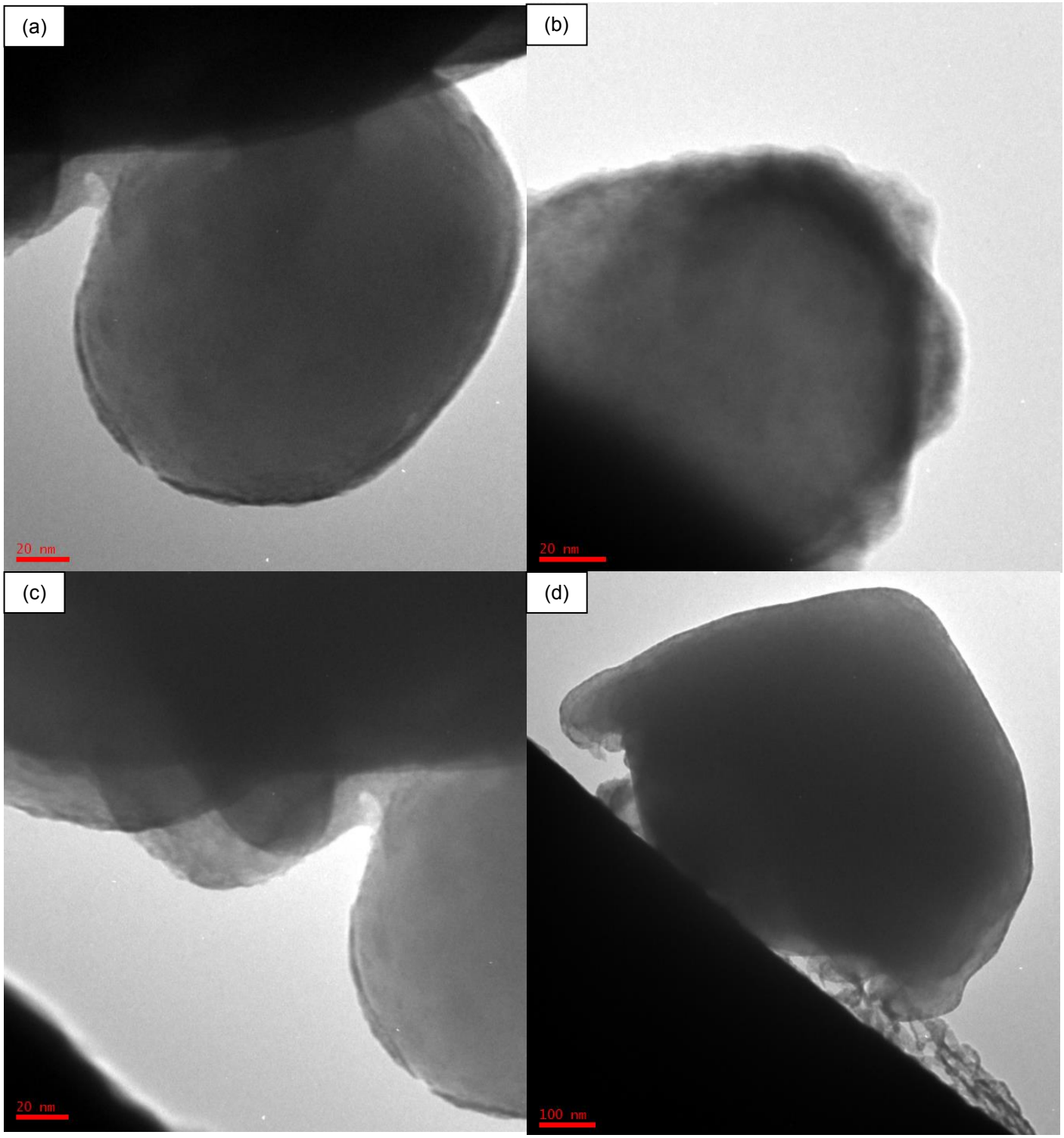

Figure 3.5: SEM images of Sf9 cells at different magnifications on Zeiss MERLIN FEG® Scanning Electron Microscope with a GEMINI II® column. **(a)-(c)** Low magnification images of individual Sf9 cells (20 nm scale bar is shown); **(d)** higher magnification image of individual Sf9 cell (100 nm scale bar is shown); **(e)-(g)** high magnification images of clusters of Sf9 cells (200 nm scale bar is shown); **(h)** high magnification image of cluster of Sf9 cells (0.2 µm scale bar is shown).

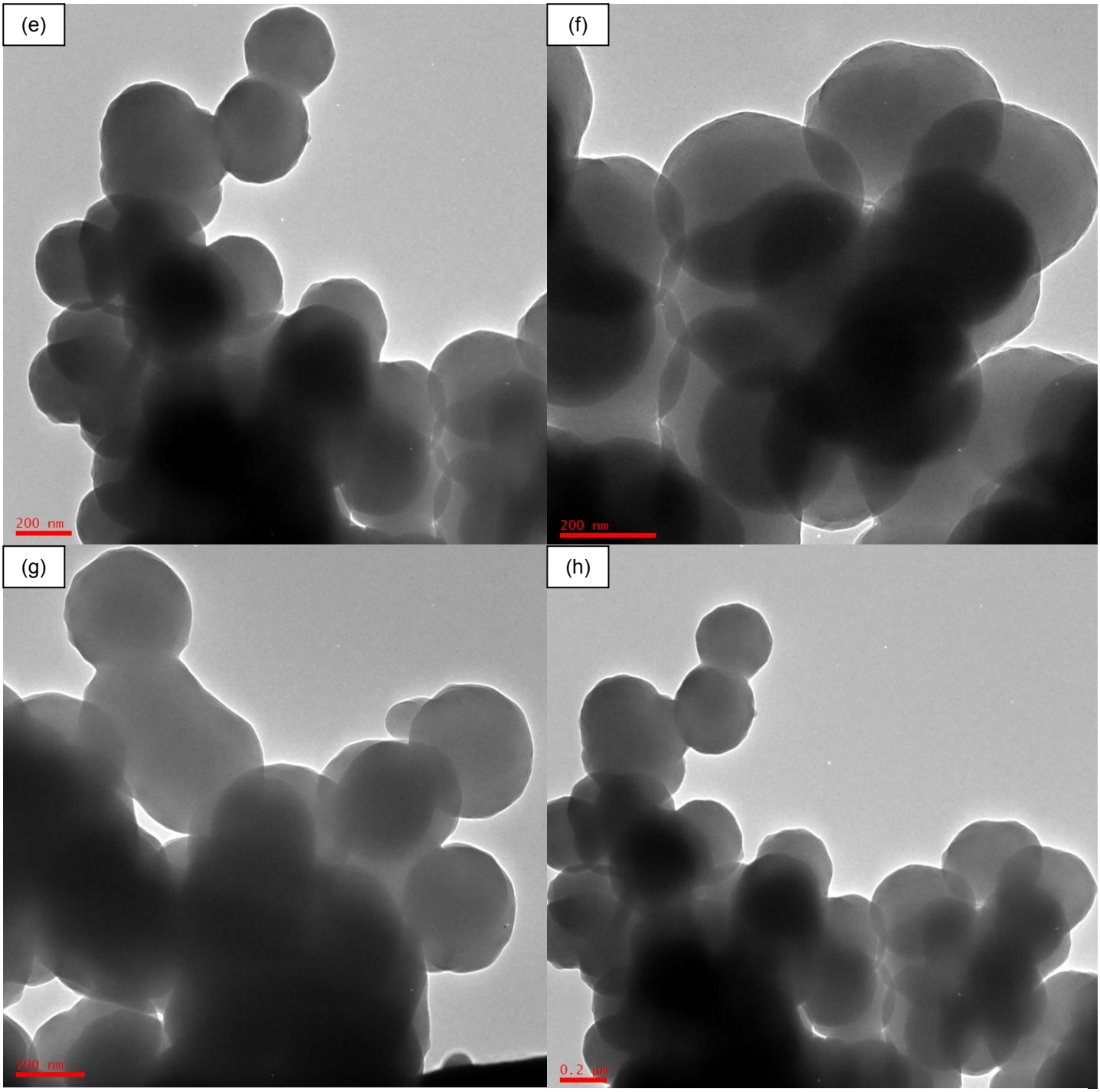

Figure 3.5: SEM images of Sf9 cells at different magnifications on Zeiss MERLIN FEG® Scanning Electron Microscope with a GEMINI II® column. **(a)-(c)** Low magnification images of individual Sf9 cells (20 nm scale bar is shown); **(d)** higher magnification image of individual Sf9 cell (100 nm scale bar is shown); **(e)-(g)** high magnification images of clusters of Sf9 cells (200 nm scale bar is shown); **(h)** high magnification image of cluster of Sf9 cells (0.2 µm scale bar is shown).

3.3.4 PCR and gel electrophoresis

PCR amplification using previously described Lepidopteran-specific baculovirus primer pairs *polh/gran*, *lef-8*, and *lef-9* (Lange *et al*. 2004) did not yield any bands when performing gel electrophoresis (Figure 3.6). Only what appeared to be primer dimers were observed below 100 bp on the 1.5% agarose gel. The presence of template DNA in the Sf9 synthesised cDNA and extracted gDNA was not verified using a ribosomal protein 18S primer pair. Therefore, the presence of a wild type baculoviral infection in the Sf9 cells could not be confirmed.

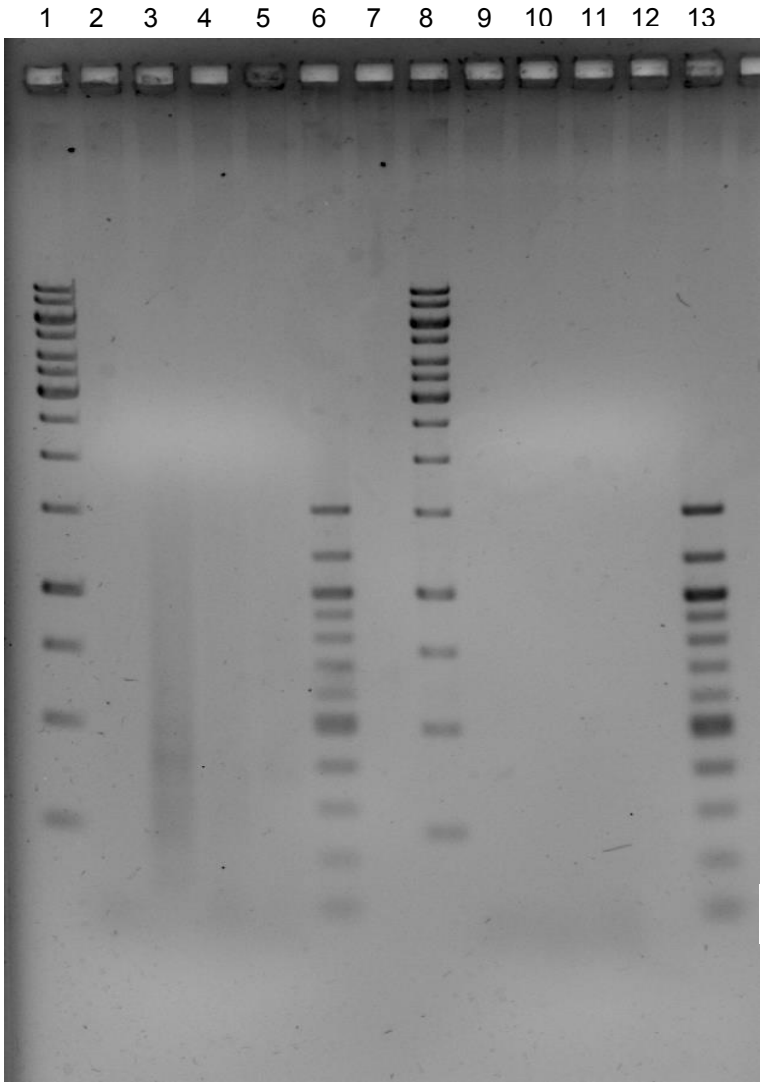

Figure 3.6: 1.5% gel with Sf9 DNA/cDNA using Lepidopteran-specific baculovirus primers. Lane 1 and 8: 1 kb ladder; Lane 2, 9: prPH primer pair with Sf9 DNA, cDNA; Lane 3, 10: prL8 primer pair with Sf9 DNA, cDNA; Lane 4, 11: prL9 primer pair with Sf9 DNA, cDNA; Lane 5, 12: 18S primer pair with Sf9 DNA, cDNA; Lane 6 and 13: 100 bp ladder.

3.4 Discussion

3.4.1 Culturing of the Sf9 cell line

The initial objective in this chapter was to use the Lepidopteran Sf9 cell line to study *D. noxia* gene expression and regulation *in vivo*. However, this proved a difficult task due to the inherent lack of cell totipotency of the purchased Sf9 cell lines [(Sf9, ThermoFisher Scientific, USA); (Sf9 CRL-1711™, ATCC®, USA)] – even after the cell line was purchased twice from two separate suppliers.

After initially suspecting yeast/fungal contamination of the adherent Sf9 cell culture (Sf9 CRL-1711™, ATCC®, USA) it was later thought to have been a wild type baculovirus infection instead, as structures resembling occluded viral particles were visible inside the cells. Unfortunately no photographs could be taken of the culture as the infrastructure did not allow for it and also to prevent further disturbances of the culture/additional contamination at that time by transporting the culture flasks to an alternative facility. However, what was observed under the microscope coincided with a published image of Sf9 cells infected with a wild-type *Autographa californica* nuclear polyhedrosis virus (Figure 3.7) (Litts 2000), where polyhedra – polymers of 33-kDa polyhedron protein – are clearly visible within individual cells.

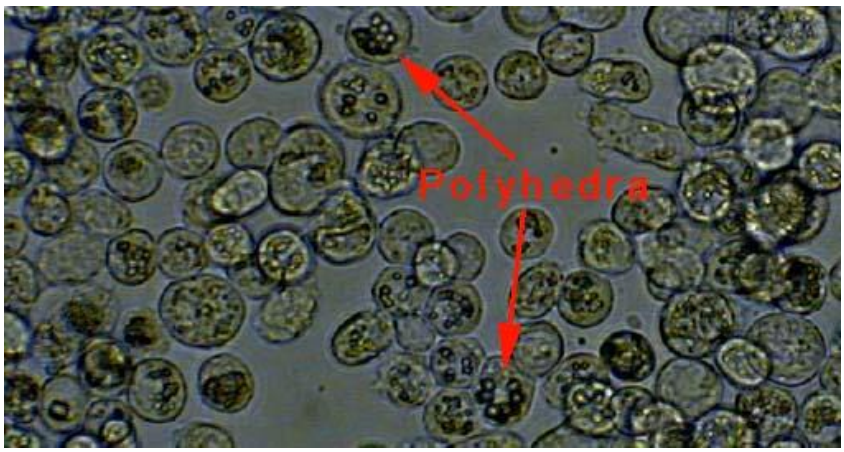

Figure 3.7: Sf9 cells infected with wild-type AcNPV (Autographa californica nuclear polyhedrosis virus). Light micrograph of Sf9 cells 4 days after a wild-type AcNPV infection occurred (Litts 2000).

Baculovirusses form part of the Baculoviridae family of viruses, consisting of 49 species divided among 4 genera (Jehle *et al*. 2006). These lytic viruses are pathogens that mainly attack insects and other arthropods (D'Amico 2016) and are recognised for their versatility and effectiveness as gene expression vectors, biological pesticides as well as vectors for mammalian cell transduction (Szewczyk *et al*. 2006; Chen *et al*. 2011, Assenberg *et al*. 2013). Despite typically possessing narrow host ranges that are often restricted to only one or a few related insect species, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), which is the most thoroughly studied baculovirus, can infect as many as 30 species from various lepidopteran genera (Clem & Passarelli 2013). Baculovirus nucleocapsids are enclosed in an envelope and rod-shaped in appearance, while circular genomes containing double-stranded DNA in varying size from about 80-180 kbp (kilobase pair) in length are present (Clem & Passarelli 2013). These viruses encode copious accessory genes involved in manipulating cellular processes like cell cycle and apoptosis (Braunagel *et al*. 1998; Clem 2007), along with host physiology and behaviour. Two dissimilar types of enveloped virions can be formed by baculoviruses, namely occlusion-derived virions (ODV) and budded virions (BV) (Figure 3.8). ODVs are fixed in large protein crystals (5-10 micron) known as occlusion bodies and control the horizontal transmission between insects, whereas BVs spreads infection from cell to cell (Clem & Passarelli 2013).

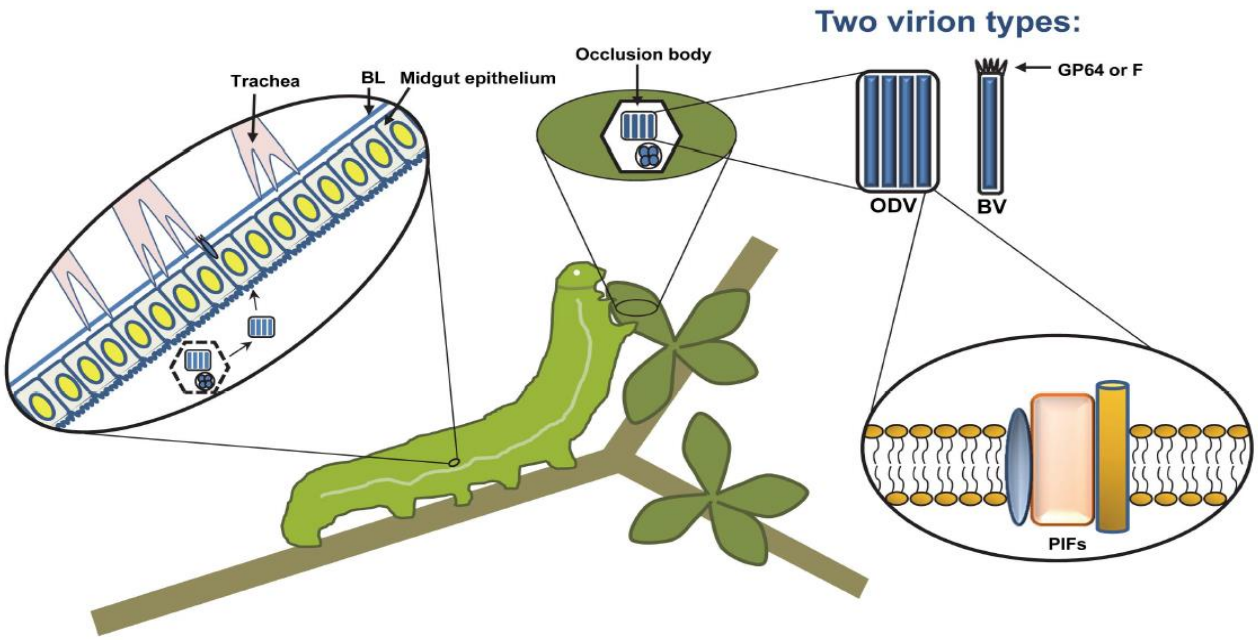

Figure 3.8: A typical baculovirus replication cycle. Nucleocapsids are formed in the nucleus of infected cells and two distinct forms of enveloped virions are produced. Firstly the budded virus (BV) is formed when single nucleocapsids leave the nucleus and bud from the cell, thus obtaining an envelope from the plasma membrane. The BV attachment and fusion protein (GP64 or F) is concentrated at one end of the virion in structures termed peplomers. Later, occlusion-derived virus (ODV) is produced when nucleocapsids gain an envelope from the inner nuclear membrane. ODV are surrounded by large proteinaceous crystals mainly consisting of a single viral protein called polyhedrin, and are termed occlusion bodies. These occlusion bodies persist in the nucleus until they are released via cell lysis. Some baculoviruses like multiple nucleopolyhedrovoruses (MNPVs), hold multiple nucelocapsids inside a single-enveloped virus particle. The baculovirus replication cycle starts when susceptible insect larva ingests viral occlusion bodies contaminating their food source. The occlusion bodies are dissolved in the highly alkaline midgut of the larva, ODV are released and attach via the PIF proteins to the microvillar membranes of midgut epithelial cells. BV are produced by infected midgut epithelial cells. Budding takes place from the basal side and tracheal epithelial cells are infected. Infection of tracheal cells seems to be one of the mechanisms that facilitates BV escape across the midgut basal lamina (BL) and dispersal of infection throughout the insect [Adapted from Clem & Passarelli 2013].

Additionally, the “infected” Grace’s Insect medium incubated with DMEM growth medium containing phenol red did not indicate fungal infection (Figure 3.4). This mixture of growth medium was incubated at 37°C, the optimal growth temperature for yeast/fungi. Phenol red is widely used in culture media as a pH indicator to identify changes of neutral to acidic pH values. Its colour shows a gradual transition from yellow to red over the pH range 6.8 to 8.2, while it turns bright pink above pH 8.2. An increase of yeast cells, due to incubation at optimal growth temperature, would have led to the acidification of the medium, which in turn would result in the medium turning yellow. However, this colour change of the medium was not observed and would therefore point to the absence of a yeast infection. Thus, it was attempted to verify the viral infection through SEM.

SEM imaging of Sf9 cells was conducted to visualise and confirm the manifestation of a viral infection during culturing. However, no viral particles were observed inside the individual or clusters of Sf9 cells (Figure 3.5). This could possibly be due to the phase of the lifecycle the baculovirus was in when the SEM sample preparation was done. Baculoviruses are known to exhibit a biphasic replication cycle in its insect host, comprising of the development of two types of virions with unrelated functions during different phases of the infection process (Monteiro *et al*. 2012). The first being occlusion-derived virions (ODVs) which displays a higher stability outside the insect host. Secondly, the budded virions (BVs) that is non-occluded and causes the systemic, cell-to-cell distribution of the virus within the insect. Baculoviral infections are characterised by three consecutive phases: early (0-6 hours post infection), late (6-24 hours post infections), and very late phase (18-24 to 72 hours post infections). ODVs are produced during the later stages of the infection and occlusion bodies are then released from the cells when they lyse (https://oetltd.wordpress.com/2014/07/17/growing-pains-the-life-cycle-of-the-baculovirus/). Therefore, it is likely that the baculovirus infection was in its late stages resulting in no visible viral particles in the infected Sf9 cells via SEM imaging. Additionally, the sample preparation would cause a loss of the occlusion bodies and the lysed infected cells, while only a few of

the non-infected cells would remain. Consequently, the possibility of a baculovirus infection of the Sf9 cell culture could not be confirmed using SEM.

In 2004, Lange *et al*. explored baculovirus diversity and aimed to establish a fast and universal tool for virus identification by investigating the available genome sequence information of lepidopteran-specific NPVs (nucleopolyhedroviruses) and GVs (granuloviruses). The resulting degenerate primers displayed successful PCR amplification of the highly conserved gene sequences – *polh/gran*, *lef-8*, and *lef-9*. PCR amplification of Sf9 cDNA and extracted gDNA using the aforementioned three Lepidoteran-specific baculovirus primer pairs was performed, but was unsuccessful in verifying the occurrence of a wild type baculovirus infection of the Sf9 cultured cells (Figure 3.6). Therefore, an alternative explanation was required as to what caused the detrimental effects on the Sf9 cell culture.

In 2014, Ma *et al*., stumbled upon the presence of a rhabdovirus in Sf9 cell lines – the first identified in the order *Lepidoptera* - while investigating the employment of a combinatorial testing approach to evaluate cell lines for unexpected viruses. Electron microscopy, using filtered supernatant from Sf9 cells, confirmed the presence of an intact virus resembling the rhabdovirus morphology. A large amount of extracellular particles were generated from the cells, therefore demonstrating that the virus most probably replicates within the cells while it seems to be constitutively produced from the Sf9 cell line due to its observed persistence (Ma *et al*. 2014). The Sf9 cell line is routinely utilised for the development and manufacturing of biological products, therefore the presence of the Sf-rhabdovirus in these cells were quite a surprising result. Whether this inherent Sf-rhabdovirus was responsible for the deterioration of the Sf9 cell line grown in this study, is uncertain. Due to time constraints and limited sample, further analysis could not be performed on the Sf9 cells.

3.4.2 Attempts to establish a *D. noxia* cell line

Since the Sf9 cell line was rendered unsuitable for the planned experiments, attempts were made to establish a primary RWA cell culture that could be utilised for expression and silencing of the selected insect transcripts. The culturing of animal cells are more complex compared to microorganisms due to their need for many more nutrients and attachment to specifically coated surfaces to enable growth (Shenoy 2007). Regardless of these hitches, various types of animal cells, including undifferentiated and differentiated cells, can successfully be cultured. Generally, three main types of cell cultures are defined – primary culture, secondary culture, and cell line/strain. Primary cultures are established when cells are surgically removed from an organism and placed into an appropriate culture environment, where they will attach, divide and grow (Shenoy 2007). Thus, primary cultures are derived from whole or dissociated tissues or organ fragments (Figure 3.9).

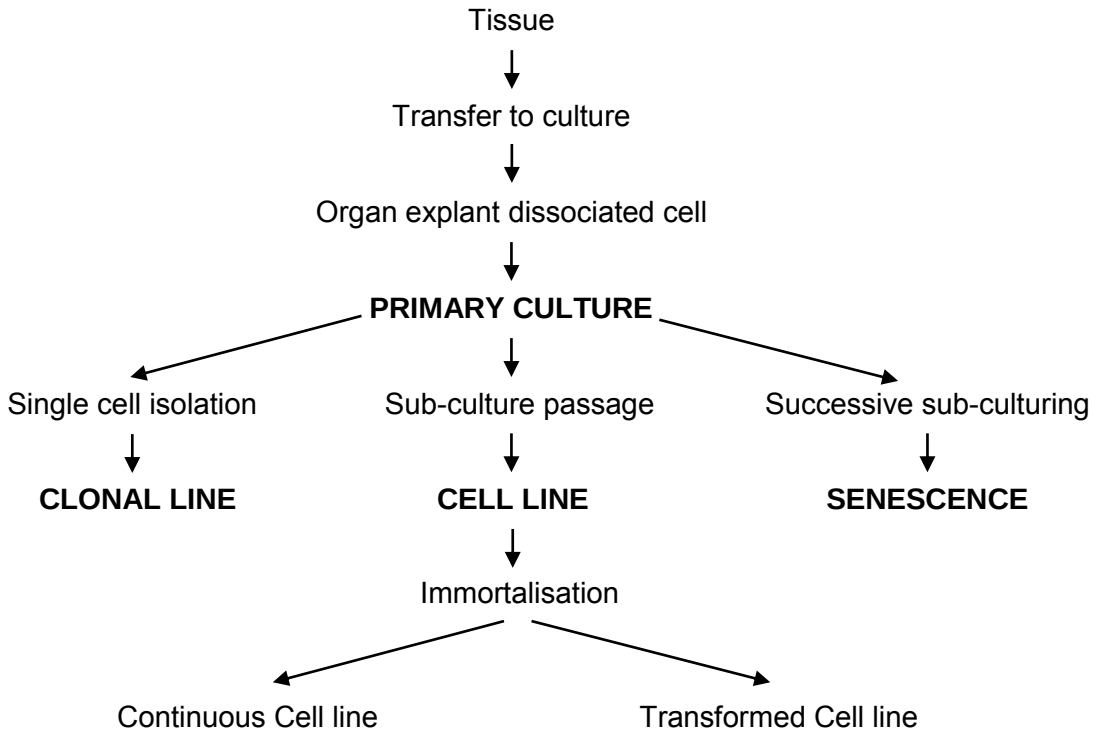

Figure 3.9: Diagrammatic representation of culturing of animal cells [Adapted from Shenoy 2007].

According to Lynn (2001), several cells are cultured from ovaries, embryos, hemocytes, imaginal discs, fat bodies, midguts, neonate larvae, cuticles, as well as the nervous, endocrine and muscular systems. Generally, embryos and further contents of the uterus are free from contamination, whereas adult tissues are prone to bacterial contamination (Shenoy 2007). When performing tissue isolation, surface sterilisation with 70% (v/v) ethanol is essential and the dissected tissue should be transferred to balanced salt solution supplemented with antibiotics or an appropriate growth medium. A need exists for primary cell cultures derived from diverse and new species with the purpose of investigating and revealing the interactions between molecules occurring in different cells (Soya *et al*. 2015). Also, to identify more effective cell lines with the capability of generating recombinant humanized proteins in eukaryotic systems (Soya *et al*. 2015). Therefore, in the present study we aimed to establish a protocol for successful generation of an RWA primary cell culture, which can be utilised in this study and for future research.

Several variations on the described protocol were tested in order to attempt the establishment of a primary cell culture from RWA embryos. Bacterial contamination seemed to be a crucial factor worth addressing. Typically, the medium would take on a milky, white appearance after incubation for 12-24 hours. This would persist even after additional anti-bacterial agent was added. As mentioned, the cultures were investigated daily and if signs of contamination were observed, the medium was replaced with fresh medium and double the dose of penicillin-streptomycin. In an attempt to combat the bacterial contamination, some adjustments were made to the original protocol. Firstly, the aphids were surface sterilised with 70% (v/v) ethanol for a longer period – at least 5 minutes – before being dissected. Secondly, the isolated embryos would be subjected to 1.6% (v/v) sodium hypochlorite (bleach) for an incubation of 10 minutes, whereafter a washing step with sterile dH_2O was repeated three times (García *et al*. 1995). However, this modification appeared to be a little too harsh for the fragile embryos and several were “lost” in the process. The embryos were also crushed in an Eppendorf tube containing Grace’s Insect Medium supplemented with

penicillin-streptomycin, but still bacterial contamination was observed. Optimisation of the protocol proved to be very time-consuming and seemed to steer the study away from the main aim.

The reason for the inability to keep the cultures sterile, may also be found in a study by Campillo *et a*l. (2015) where it was reported that members of *Enterobacteriaceae* were consistently isolated from sterile artificial diets post RWA feeding. Artificial diets subjected to probing through a parafilm membrane via the RWA stylets contained bacteria, while sterile diets without RWA exposure were bacteria-free. Bacteria were also isolated from both crushed RWA bodies and wheat subjected to aphid feeding (Campillo *et al*. 2015). A subset of the above-mentioned isolates was grouped to the *Erwinia-Pantoea* clade. Therefore, proposing that bacteria are naturally present in the RWA, seemingly within the stylet. This could provide a possible explanation for the constant bacterial contamination observed when attempting to establish a primary RWA cell culture. Instead of focussing on the potential inherent bacteria in the RWA, it was decided to explore and follow alternative avenues in order to silence the selected insect transcripts, as presented in Chapter 4.

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Chapter 4:

In vivo RNAi of selected insect transcripts in RWA

4.1 Introduction

An intimate association exists between plants and insects. In order to survive and reproduce, pests like the phloem-feeding aphids usually require close interaction with their host plants, during which effectors are transported inside the plant to modify host cell processes (Rodriguez & Bos 2013). According to Rodriguez and Bos (2013), "effectors" refer to "proteins or small molecules secreted by plant-associated organisms that alter host cell structure and function." Since the effectors are expressed in the salivary glands of insect pests, such as aphids, it is hypothesised to be secreted with the saliva whereby delivery into the host plant commences (Rodriguez & Bos 2013). The Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov, Hemiptera: Aphididae), is regarded as one of the most destructive and widely distributed insect species in the world (Heyns 2005; Turanli *et al*. 2012). Owing to its swift population growth, this wheat pest inflicts major damage in South Africa and various other countries (Botha *et al*. 2014).

In agricultural systems, crop damage due to insect pests is primarily characterised by a loss in yield or quality which in turn holds financial implications for farmers. Over the past decades, the utilisation of chemical insecticides has been the primary mode of RWA infestation management. Due to leaf rolling, the RWA nestles itself in an optimum environment which provides protection from contact insecticides (Miller *et al*. 1994). Additionally, it has been found that several aphid species have the ability to develop resistance against chemical insecticides (Sapountzis *et al*. 2014). In order to decrease the use of expensive and potentially hazardous insecticides, RWA resistant wheat cultivars have been forming the basis of an integrated pest management programme. However, due to the on-going arms race between insects and plants - such as the RWA and wheat - insects have acquired the ability to develop new biotypes (Jankielsohn 2013).

Biological control agents can also be used as a means of reducing RWA population levels, but as this is an introduced pest in South Africa no naturally occurring native predators are

present, causing RWAs to thrive on susceptible wheat. Another constraint of biological control agents lies in their proneness to environmental conditions. Consequently, it has become a vital task to discover and develop alternative methods for aphid control and this can be achieved through a better understanding of their biology.

RNA interference (RNAi) is an up and coming biotechnology tool with applications in numerous fields and the ability to strongly facilitate the advancement of molecular biology (Jung & Zhao-jun 2014). More importantly, RNAi has gained accreditation for efficaciously silencing specific or fatal genes in insects and therefore can be considered as a potential newfound pest control strategy (Baum *et al*. 2007; Mao *et al*. 2007; Huvenne & Smagghe 2010). Nonetheless, an efficient and opportune mode of dsRNA delivery into the organism is still a restraining factor in its success. Different techniques have been tested to address this concern, including microinjection (Bettencourt *et al*. 2002; Tomoyasu & Denell 2004; Ghanima *et al*. 2007), soaking, and oral feeding using an artificial diet (Eaton *et al*. 2002, Turner *et al*. 2006, Baum *et al*. 2007, Mao *et al*. 2007, Chen *et al*. 2008, Tian *et al*. 2009). Feeding dsRNA via artificial diet is currently the most appealing delivery method due to its convenience and ability to be easily manipulated (Yang *et al*. 2011). Insects are subjected to less damage than with microinjections as this is a more natural mode of dsRNA transfer into the body of the insect (Chen *et al*. 2010). This method is especially favourable when working with very small insects, such as the RWA, because they are challenging to manipulate by microinjection. However, incomplete silencing has been observed using feeding by artificial diet and thus a greater amount of material used for delivery is needed (Yang *et al*. 2011). Furthermore, diverse sensitivities to RNAi molecules have been detected in different insect species as a result of oral delivery (Walshe *et al*. 2009). An adaptation to the feeding with artificial diet approach, concerns the injection of siRNA into the vein of plant leaves fed on by aphids. The injection method allows the aphids to undergo the feeding trial in a more natural habitat in comparison to feeding on an artificial diet in a tube. Thus, in the present study

feeding with an artificial diet as well as injection of plant leaves with siRNA will be used to investigate *in vivo* silencing of the *C002* and *14-3-3 ε* transcripts in RWA.

C002 is described as an aphid-specific protein with unknown function that is present in watery saliva (Yong *et al*. 2014) and is delivered inside the host plant tissue during feeding (Mutti *et al*. 2008). It appears to be primarily expressed in the salivary glands of pea aphids (*Acyrosiphon pisum*) and green peach aphids (*Myzus persicae*) (Yong *et al*. 2014). However, more recently semi-quantitative PCR analysis indicated low expression of *C002* in the guts of *A. pisum* (Mutti *et al*. 2006; Pitino *et al*. 2011). Through the injection of siRNA molecules into the abdomen of pea aphids, Mutti *et al*. (2006; 2008) demonstrated that the C002 protein fulfils a vital role in some aspects of the foraging and feeding behaviour of aphids. The saliva of *A. pisum* and *M. persicae* have been shown to contain C002 protein (Carolan *et al*. 2009; Harmel *et al*. 2008), whereas phylogenetic analysis indicated that this gene is fast-evolving in aphids, while it is not found in other insects (Ollivier *et al*. 2010). Research pertaining to *C002* has mainly been conducted on *M. persicae* and *A. pisum*. Thus, taking into account all above mentioned results, *C002* was included in the present study to investigate its role in RWA-wheat interaction.

The 14-3-3 epsilon (ε) protein belongs to the highly conserved 14-3-3 protein family, which partakes in the regulation of signal transduction pathways, adhesion, apoptosis, cellular proliferation, survival and differentiation (Mhawech 2005). This class of proteins have the ability to interact with more than 200 target proteins through phosphoserine-dependent and phosphoserine-independent approaches (Mhawech 2005). However, little is known regarding the consequences of these interactions and is therefore the subject of continuing studies. Cloete (2015) suggested that 14-3-3 ε protein might potentially be a protein elicitor present in RWA saliva, but further research needs to be conducted to investigate this notion. Thus, *14-3-3 ε* forms part of this study to determine its role in the interaction between RWA and its host plant wheat.

4.2 Materials and methods

4.2.1 Aphid rearing

The aphids used during this part of the study included South African *D. noxia* biotypes SA1 and SAM. The SA1 biotype was obtained from a separate colony that was established using parthenogenetic females collected in the field at the ARC-Small Grain Institute in Bethlehem, South Africa. This biotype was maintained on a RWA susceptible wheat cultivar, Tugela. The South African Mutant biotype (SAM) was acquired by selective pressure due to long-term force-feeding on resistant germplasm in the laboratory (Van Zyl & Botha 2008) and was sustained on the resistant wheat cultivar, TugelaDN (Dn1 containing).

4.2.2 Aphid sampling, RNA extraction and cDNA synthesis

SAM and SA1 aphids were collected from the Cereal Genomics Laboratory at the Department of Genetics, Stellenbosch University. Whole aphids were ground in liquid N_2 using a micropestle and total RNA was isolated with In-Column DNase I digestion according to the manufacturer's instructions using the RNeasy Mini Kit (Qiagen, USA). The purity and integrity of the isolated RNA was assessed through 1.5% (m/v) agarose gel electrophoresis (TAE buffer, pH 8) using ethidium bromide staining (2.5 µg/ml) as well as using absorbance at 260 nm and 280 nm in a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). The iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA) was used for cDNA synthesis (15 µl of RNA added to 20 µl reaction mixture).

4.2.3 Primer designing

Geneious Software (Version 8.0, Biomatters, New Zealand) was used to identify the homologs of four insect salivary secretion proteins (listed in Table 4.1), previously identified by Cloete (2015).

NCBI's BLAST platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al*. 1990) was then used to perform homology searches between the RWA coding sequences (CDSs)

obtained from Geneious and *Spodoptera frugiperda.* Primer pairs targeting the selected insect transcripts were designed using NCBI's Primer-BLAST (Ye *et al*. 2012) (Table 4.2). EcoRI restriction sites were added to the 5'-ends of both the forward and reverse primers for cloning purposes. Underlined sequences highlighted in orange, indicate the EcoRI restriction recognition site, while underlined sequences in blue show the leader sequence.

Table 4.1: List of salivary protein transcripts identified as potential effectors (Cloete 2015).

Transcript	Protein	NCBI* Accession No.
***Diuraphis noxia C002* gene**	C002	JN092369.1
***Acyrthosiphom pisum* apolipophoris protein LOC100159010**	Apolipophorins protein	XM_008185734.1
***Acyrthosiphom pisum* uncharacterized LOC100169243**	Uncharacterised	XM_001943863.3
Acyrthosiphom pisum 14-3-3 protein epsilon	14-3-3 protein epsilon	NM_001162004.2

*National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/)

Table 4.2: Primer pairs targeting the selected insect transcripts.

Transcript	Primer pair
C002	**Forward:** 5'- TAAGCA GAATTC CTGAGAAAATGGGCTCCGAC-3' **Reverse:** 5'- TAAGCA GAATTC GCACTCCTTGCCATCTTGGT-3'
LOC100169243	**Forward:** 5'- TAAGCA GAATTC TCGCAATACGCAACAATAGCA-3' **Reverse:** 5'- TAAGCA GAATTC TCAGTCATTTCGAATTCAATGTTGT-3'
Apolipophorins protein	**Forward:** 5'- TAAGCA GAATTC TTACAGACGACCACAGACGA -3' **Reverse:** 5'- TAAGCA GAATTC TGCAAAATATCCTTCGGGTCCA-3'
14-3-3 protein epsilon	**Forward:** 5'- TAAGCA GAATTC AGTTCCTGTGGAAGGCGAAG-3' **Reverse:** 5'- TAAGCA GAATTC CTCTGGAAATGGCCACCACT-3'

4.2.4 Cloning and sequencing

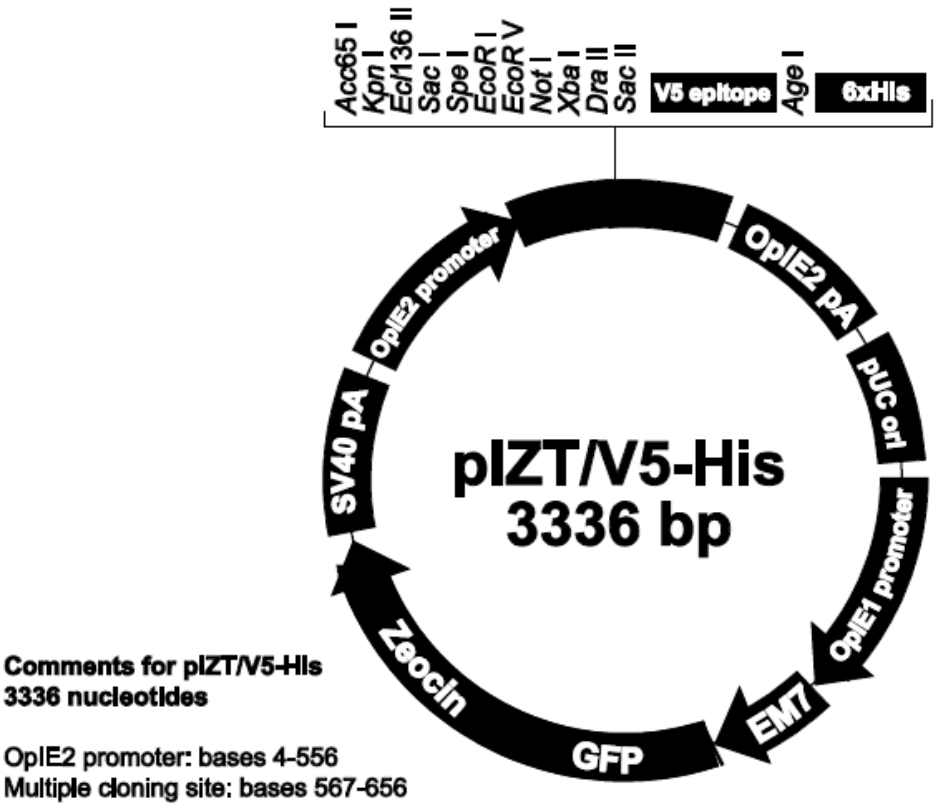
Cloning of the four transcripts was performed using *E.coli* DH5α™ cells (Invitrogen, USA) and the pIZT/V5-His vector (Invitrogen, USA) (Figure 4.1), which was initially obtained to perform the expression and silencing of the selected transcripts in the Sf9 cell line (Chapter 3). EcoRI restriction digestion was done, followed by overnight ligation. Transformants were selected on Low Salt Luria broth (LB) plates (1.0% m/v Tryptone; 0.5% m/v Yeast Extract; 0.5% m/v NaCl; pH 7.5) containing 25–50 µg/mL Zeocin™ as prescribed by the InsectSelect™ Glow System Manual (Invitrogen 2012). Colony PCRs and agarose gel electrophoresis were performed to verify positive transformants (Zaayman *et al*. 2009). The Plasmid miniprep kit from Qiagen (USA) was used to isolate plasmid DNA of the positive transformants, which was sent for sequencing at the Central Analytical Facilities (CAF) (Stellenbosch). Two colonies of each transcript were sent for sequencing using the opIE2 forward and reverse primer pair, in order to verify whether the correct sequences were amplified.

4.2.5 Design and synthesis of *C002* and *14-3-3 ε* siRNA

The Custom Dicer-Substrate siRNA (DsiRNA) design tool of Integrated DNA Technologies (IDT) was used to design the *C002* and *14-3-3 ε* siRNA oligonucleotides (Table 4.3).

Table 4.3: *C002* and *14-3-3 epsilon* siRNA oligonucleotide sequences designed on DsiRNA (IDT).

Transcript	siRNA oligonucleotide sequences
C002	**Sequence 1-** CAA CUC CAG AGA UUC GUG ACU UUA UCA **Sequence 2-** AUA AAG UCA CGA AUC UCU GGA GUU GUC
14-3-3 ε	**Sequence 1-** GAG CUA UAA AGA UUC CAC ACU UAU AAU **Sequence 2-** UAU AAG UGU GGA AUC UUU AUA GCU CUC



Comments for pIZT/V5-His
3336 nucleotides

OpIE2 promoter: bases 4-556
Multiple cloning site: bases 567-656
V5 epitope: bases 663-704
6xHis tag: bases 714-731
OpIE2 polyadenylation sequence: bases 749-878
pUC origin: bases 947-1620
OpIE1 promoter: bases 1669-1960
EM7 promoter: bases 1975-2033
GFP-Ble fusion: bases 2067-3140
  GFP ORF: bases 2067-2771
  Zeocin resistance (*ble*) ORF: bases 2772-3140
SV40 early polyadenylation sequence: bases 3210-3336

Figure 4.1: pIZT/V5-His vector (3336 bp). This vector contains the *OpIE2* promoter for constitutive expression, the Zeocin™ resistance gene for rapid selection of stably transfected cell lines and the C-terminal V5 epitope and polyhistidine (6xHis) sequence for detection with Invitrogen's Anti-V5 Antibody and rapid purification with nickel-chelating resin. Additionally, the pIZT/V5-His vector expresses a fusion of the green fluorescent protein and the Zeocin™ resistance protein (Zeo-GFP), which permits rapid selection of stably transfected cell lines with Zeocin™ and confers a fluorescent phenotype that simplifies identification of transfected cells (Invitrogen 2012).

4.2.6 Feeding with artificial diet trial

4.2.6.1 Artificial diet

An artificial diet was prepared (Table 4.4), consisting of a carbon source (sucrose) at an optimum concentration of 20%; a nitrogen source (methionine, leucine and tryptophan); and a salt ($MgCl_2.6H_2O$) (Bahlmann 2005). The diet was buffered to pH 7.0 through the addition of a 100 mM K_2HPO_4 solution, filter sterilised and stored at 4˚C.

Table 4.4: Composition of aphid artificial diet (adapted from Bahlmann 2005).

Components	Volume (g)*
L-amino acids	
Methionine	0.10
Leucine	0.20
Tryptophan	0.10
Sucrose	20.00
$MgCl_2.6H_2O$	0.20

*pH adjusted to 7.0 with 100 mM K_2HPO_4, and ddH_2O added to make 100 ml of diet.

4.2.6.2 Feeding site setup

Custom-made open ended glass tubes (2 cm x 1.5 cm) were used to establish a feeding site for the aphids. The top and bottom open ends of the tubes were covered with stretched Parafilm [Figure 4.2 (a)]. The first membrane of the bottom end was carefully stretched to a tenth of its original thickness and placed over the end of the tube. A volume of 40 µl of the artificial diet was then added to the taut surface and covered with a second stretched membrane. Adult SAM aphids were added to the tubes using a thin, soft brush and the top end was then covered with stretched parafilm. The tubes were positioned onto a yellow

piece of paper to act as an attractant to the aphids and maintained at 25±1˚C with a 12:12 (L:D) photoperiod [Figure 4.2 (b)].

4.2.6.3 Fecundity trial

SAM aphids were placed in a petri dish and subjected to overnight incubation at 4˚C to induce starvation. Specialised sieves were used to select aphids based on size. The selected SAM aphids were placed on the artificial diet (without dsRNA) for 3 days to become accustomed to feeding on artificial medium. After 3 days the aphids were removed and incubated at 4˚C while tubes were prepared with artificial medium containing siRNA. For each transcript as well as the control, 6 tubes were prepared containing artificial medium and 1 μg/μl siRNA (control contained water instead of siRNA) to a final volume of 40 μl. One SAM aphid was placed in every tube (n=6). RNAi treatment commenced and the aphids were allowed to feed on artificial diet containing siRNA. Fecundity of the aphids were monitored by counting the amount of nymphs produced in each tube starting 24h after initial incubation and continued until 120h.

4.2.6.4 Sampling trial

For the sampling trial, some adjustments were made to the fecundity trial protocol. For each transcript as well as the control, 3 feeding tubes were prepared containing artificial medium and 1 μg/μl siRNA (control contained water instead of siRNA) to a final volume of 40 μl. Eight SAM aphids were placed in every tube (n=24) to ensure that suffient aphids were available for sampling at each time-point. RNAi treatment commenced and the aphids were allowed to feed on artificial diet containing siRNA. At all of the time points (6h, 24h, 48h and 72h), one SAM aphid were carefully sampled from each tube (3 independent biological replicates for each treatment and control) using a thin, soft brush and placed in an Eppendorf tube (a total of 12 aphids sampled). Sampling started at 6h after initiation, followed by 24h, 48h, and 72h. The Eppendorf tubes containing the sampled aphids were flash frozen using liquid N_2 and stored at -80°C until RNA extraction could be performed.

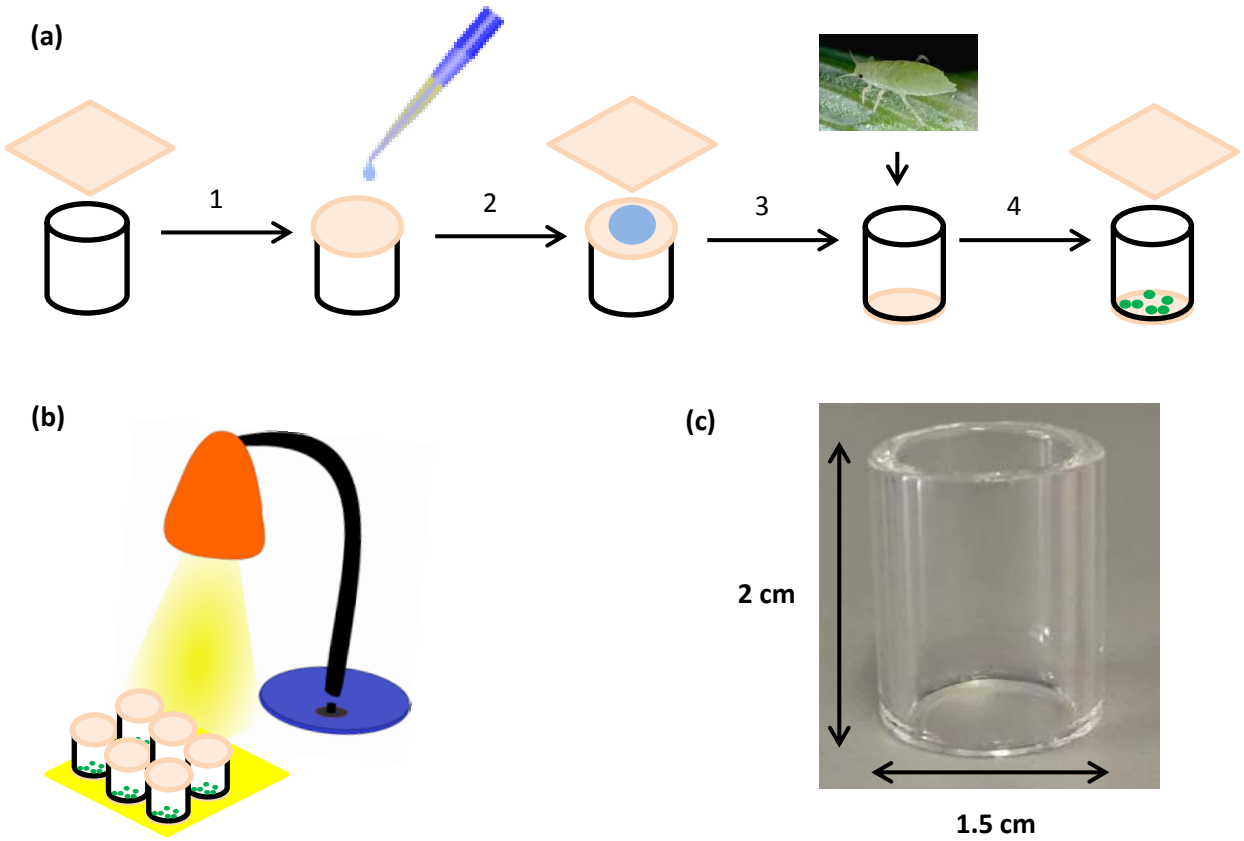


Figure 4.2: Artifial diet feeding site setup. **(a)** Custom-made open-ended glass tubes were used to conduct the feeding with artificial diet trials. One open end was covered with stretched parafilm (1) and 40 µl of artificial medium was pipetted onto this layer (2). A second layer of stretched parafilm was carefully placed over the first layer containing the artificial medium (3). Adult aphids were added to the tube (4) and the other open end was covered with stretched parafilm to allow for air flow, but preventing the aphids from escaping. **(b)** The tubes were positioned on a yellow paper with the feeding site area at the bottom, under a light connected to a timer to maintain a 12:12 (LD) photoperiod. **(c)** The open-ended glass tubes were 2 cm x 1.5 cm in size.

4.2.7 siRNA injection into wheat

4.2.7.1 Injection site setup

The leaves of six 50 day old wheat plants (Gamtoos R) were injected using a Hamilton syringe and 1 mm needles. A volume of 1 µl of 1µg/µl siRNA dissolved in 10 mM Tris, pH 7.0 and then injected into the vein at the bottom of the third leaf, at three locations 10 mm apart

resulting in a total of 3 μg injected siRNA into each leaf (three leaves per plant, n=3). The control leaves were injected with 10 mM Tris-HCl, pH 7.0, only.

4.2.7.2 Fecundity trial

The custom made aphid cages [Figure 4.3 (b)] consisted of 2 equally sized pieces of double-sided tape stuck together (approximately 25 mm x 12 mm with a joined height of 3 mm) with a 5 mm diameter hole punched in the middle [Figure 4.3 (a)] were used to assess aphid response during fecundity trial. The top part of the hole was covered with gauze to ensure air flow and that aphids remained within the cage. Specialised leaf clips were used to position the custom made aphid cages at the injection sites on the leaves [Figure 4.3 (f)]. Three cages were placed adjacent to one another, with two cages containing one aphid each (to monitor fecundity, n=2) and the third 6 aphids (for sampling, n=6) [Figure 4.3 (e)]. Three leaves were injected per treatment as well as the control (n=6 for fecundity and n=36 for sampling, for each treatment and the control). The aphids subjected to the experiment were selected based on size using specialised sieves, put in a petri dish and placed in the fridge overnight prior to injection to induce starvation.

RNAi treatment commenced and the aphids were allowed to feed on the leaves adjacent to the siRNA injection sites. Aphids were monitored for nymph production in the cages containing only one aphid, starting at 24h after injection and continued at 48h, and 72h.

4.2.7.3 Sampling trial

RNAi treatment commenced and the aphids were allowed to feed on the leaves at the siRNA injection sites. At all of the time points, one SAM aphid were carefully sampled from each injection site [3 independent biological replicates for each treatment (n=3) and control (n=3)] using a thin, soft brush and placed in an Eppendorf tube. The eppendorf tubes containing the sampled aphids were flash frozen using liquid N2 and stored at -80°C until RNA extraction could be performed. Sampling started at 6h after injection, followed by 24h, 48h, and 72h.

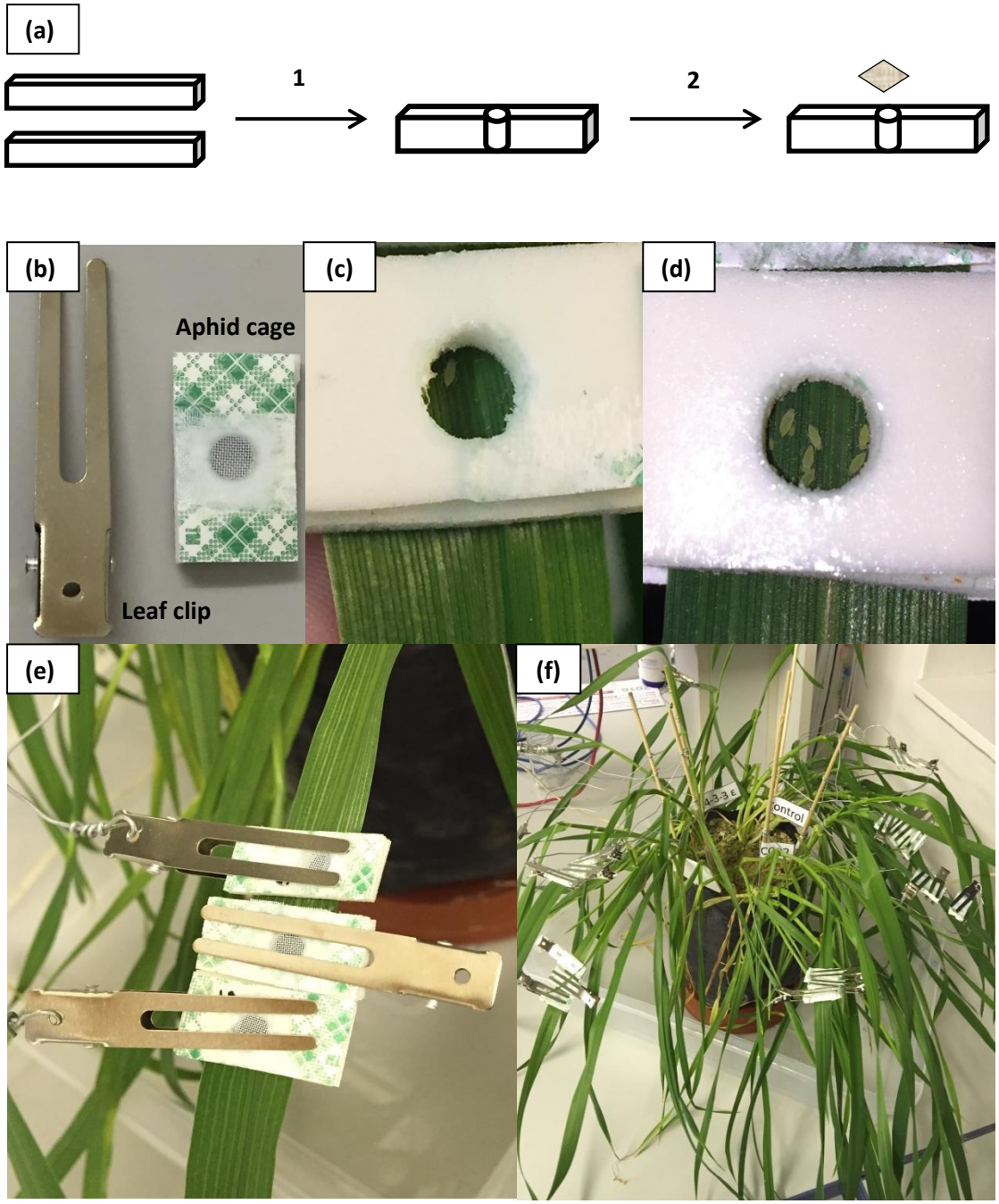


Figure 4.3: Injection of siRNA into wheat leaves setup. **(a)** Custom made aphid cage were constructed consisting of two equally sized layers of double-sided tape (1) stuck together and a hole punched in the middle (2). A piece of gauze was placed over the top part of the hole to restrict aphids to the cage. **(b)** A leaf clip and constructed aphid cage. **(c)** Two of the three cages on each leaf initially contained one adult aphid each and **(d)** the third six adult aphids. **(e)** The three aphid cages were secured on each leaf near/adjacent to the injection sites using the leaf clips. **(f)** Sticks and wire were used to support the leaves.

4.2.8 Gene expression analysis using real time (RT)-qPCR

4.2.8.1 RNA extraction

Total RNA was extracted from the 6h, 24h, 48h, and 72h sampled SAM aphids [feeding with artificial diet trial (n=3) and injection trial (n=3)] using the RNeasy Mini Kit (Qiagen, USA). The iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA) was used for first strand cDNA synthesis and samples were quantitated via Qubit analysis [Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, USA)] at CAF Stellenbosch.

4.2.8.2 Primers

The RT-qPCR primers for *C002* and *14-3-3 ε* were designed on Geneious Software (Version 8.0, Biomatters, New Zealand) (Table 4.5) and ordered from IDT (USA). Sequences of the reference genes, ribosomal proteins L27 (Sinha & Smith 2014) and L32 (Shakesby *et al*. 2009), were acquired from literature. Optimal parameters for each primer set were determined using the Taguchi method (Thanakiatkrai & Welch 2012). Melt curve analysis was used to verify whether the primers amplified single products and to check if primer dimers were observed.

Table 4.5: Primers for amplification and RT-qPCR of selected genes in RWA.

Primer	Tm (°C)	Sequence
C002-F	57.7	5'-TCA AGG AGC CCC GTA TGA GA-3'
C002-R	57.8	5'-CCA TCT TGG TGG GAG CTC TG-3'
14-3-3-F	55.9	5'-CGA ACA GGC GGA AAG ATA CG-3'
14-3-3-R	57.4	5'-CAC GTC GAG CTC CAA TCA CA-3'
L27-F	55	5'- ACC AGC ACG ATT TTA CCA GAT TTC-3'
L27-R	59.2	5'-CGT AGC CTG CCC TCG TGT A-3'
L32-F	56.9	5'-CGT CTT CGG ACT CTG TTG TCA A-3'
L32-R	54	5'-CAA AGT GAT CGT TAT GAC AAA CTC AA-3'

4.2.8.3 Run setup

All qPCR reactions were prepared and run in Thermo Scientific 96-Well Semi-Skirted Flat Deck PCR Plates sealed with Thermo Scientific Adhesive PCR Plate Sealing Sheets on the Bio-Rad CFX96 Real-Time System (Bio-Rad, USA). For the *C002* cDNA samples obtained in the feeding with artificial diet in tubes, Thermo Scientific Luminars Color HiGreen qPCR Master Mix (Thermo Fischer Scientific, USA) was utilised and each amplification reaction contained 2 µl of cDNA template (final stock concentration of 0.5 ng), 5 µl of 2X Luminaris Color HiGreen qPCR Master Mix, and varying volumes of each specific primer (10 pmol/µl) (Table 4.6) in a final volume of 10 µl. For the *14-3-3 ε* samples obtained from the feeding with artificial diet in a tube as well as all the samples from the injection trial, the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, USA) was utilised. The amplification program for all reactions were as follows: Initial denaturation (50°C) for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation (95°C) for 15 seconds, annealing (primer Tm) for 30 seconds and extension (72°C) for 30 seconds. Fluorescence data were read and collected at the end of each annealing/extension step and a melt curve analysis was done after the last cycle using a temperature gradient from primer Tm to 95°C and a ramp speed of 0.5°C every 5 seconds. All samples were run in triplicate (n=9) with one NTC (non-template control) and a standard curve ranging from 2 ng – 0.125 ng using SAM 0h control cDNA.

Table 4.6: Quantitative RT-PCR amplification reaction components using Thermo Scientific Luminars Color HiGreen qPCR Master Mix at 10 µl reactions.

Component	*C002*	*14-3-3 ε*	*L27*	*L32*
2X Luminaris Color HiGreen qPCR Master Mix	5 µl	5 µl	5 µl	5 µl
10 µM Forward Primer*	0.3 µl	0.6 µl	0.4 µl	0.6 µl
10 µM Reverse Primer*	0.3 µl	0.6 µl	0.6 µl	0.6 µl
Template DNA (including yellow buffer)	2 µl	2 µl	2 µl	2 µl
Water, nuclease-free	2.4 µl	1.8 µl	2 µl	1.8 µl

***Final primer concentration ranging from 0.3 µM – 0.6 µM after individual optimisation.

4.2.8.4 Statistical analysis

Gene expression values were standardised across three independent replicates, together with each sample being amplified in triplicate (n=9). Relative transcript abundance was standardised to the average expression of the SAM control aphids sampled at 6h after feeding on artificial medium without siRNA and normalisation was done against the expression of *L27* and *L32* in each sample (Pfaffl 2001). Student t-tests, to determine whether the differences in relative expression values are statistically significant, were performed on SigmaPlot® 7.0 2001 (Sigma Aldrich, USA) and $p<0.05$ was used as the level of significance.

4.3 Results

4.3.1 Cloning and analysis of sequencing data

During the cloning process, transformants were selected on Low Salt LB plates containing 25–50 µg/mL Zeocin™ as previously described. Positive transformants were observed for

C002 [Figure 4.4 (a)], *14-3-3 ε* [Figure 4.4 (b)], and the apolipophorins protein [Figure 4.4 (c)]. However, the LOC100169243 uncharacterised protein displayed great difficulty in being successfully cloned into the pIZT/V5-His vector and after several attempts it was decided to exclude this transcript from the study as no positive transformants could be produced on the colony plates [Figure 4.4 (d)].

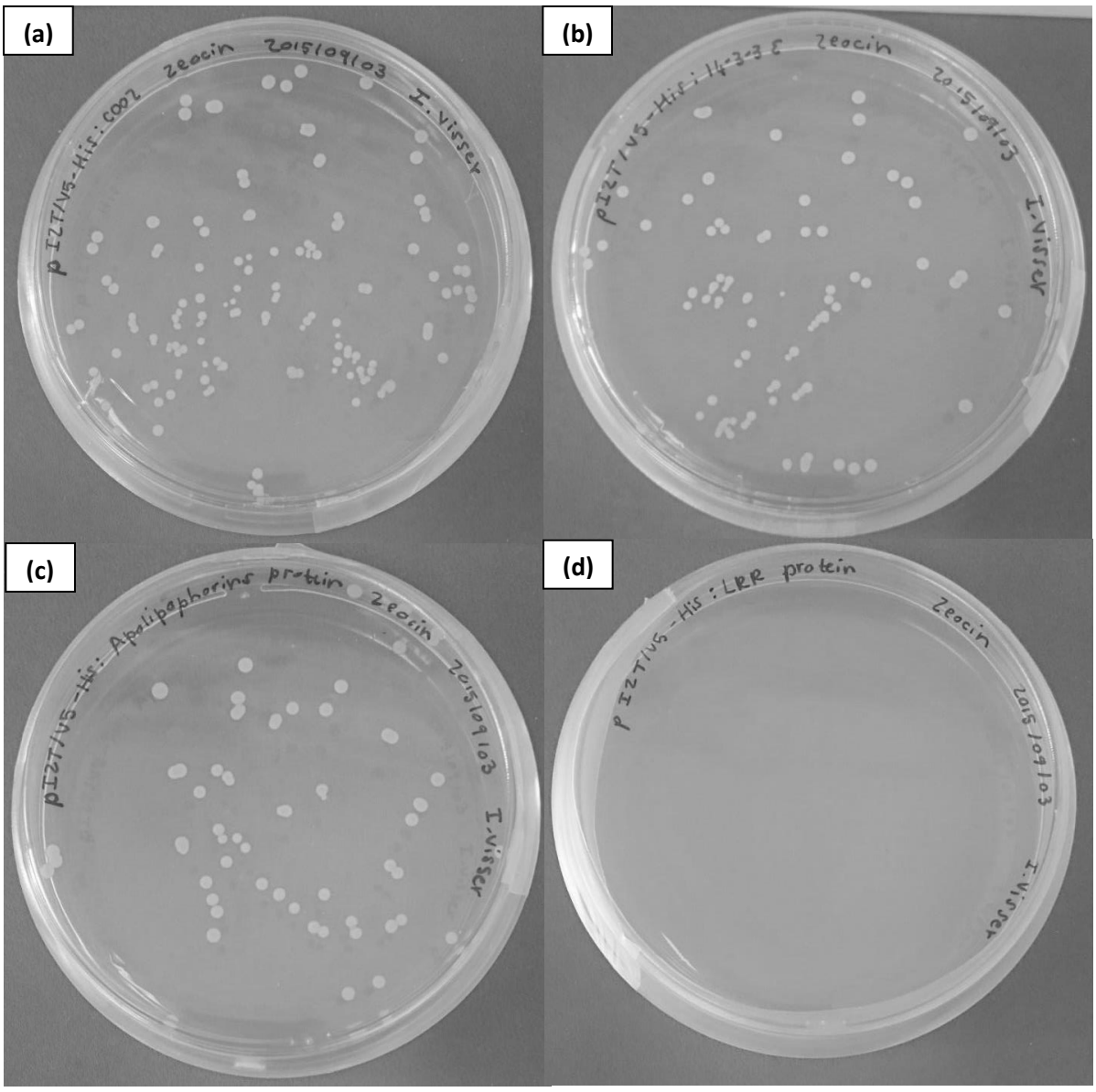


Figure 4.4: Colony plates of cloned transcripts in pIZT/V5-His vector. Positive transformants observed for *C002* **(a)**, *14-3-3 ε* **(b)**, and apolipophorins protein **(c)**. No colonies present on the LOC100169243 uncharacterised protein plates **(d)**.

Geneious Software (Version 8.0, Biomatters, New Zealand) was used to analyse the sequencing results obtained from cloning the remaining three transcripts of interest – *C002* [Figure 4.4 (a)], *14-3-3 ε* [Figure 4.4 (b)], and apolipophorins protein [Figure 4.4 (c)]. For each transcript a sequence search against the SAM genome [SAM v1.0 – Genes + SA1 SNPs (DNA)] was performed and the results indicated that *C002* [Figure 4.5 (a)] aligned well with the mRNA sequence of *Diuraphis noxia C002* gene [GenBank: JN092369.1 (Figure A1)]. Therefore it was concluded that the designed *C002* primer pair is capable of amplifying the correct sequence and could be used during the remainder of the study.

While performing the sequence alignment on Geneious utilising the *14-3-3 ε* and apolipophorins protein data obtained via cloning and subsequent sequencing, no significant alignment with the genome of the RWA was observed for either of the transcripts. This could be due to non-specific primers or could suggest that these previously identified insect transcripts are not present within the sequenced genome of the RWA. However, further investigation using the *Acyrthosiphon pisum* 14-3-3 protein epsilon [NCBI Reference Sequence: NM_001162004.2 (Figure A2)] mRNA sequence as a reference, indicated that *14-3-3 ε* is indeed present in the RWA's genome as scaffold g19560.t1 displayed significant alignment with this transcript [Figure 4.5 (b)]. This scaffold was thus used for subsequent primer- and siRNA designing for *14-3-3 ε*. Several attempts resulted in no significant alignment to apolipophorins, and it was decided to exclude the apoliphophorins protein from further experimental procedures and analysis.

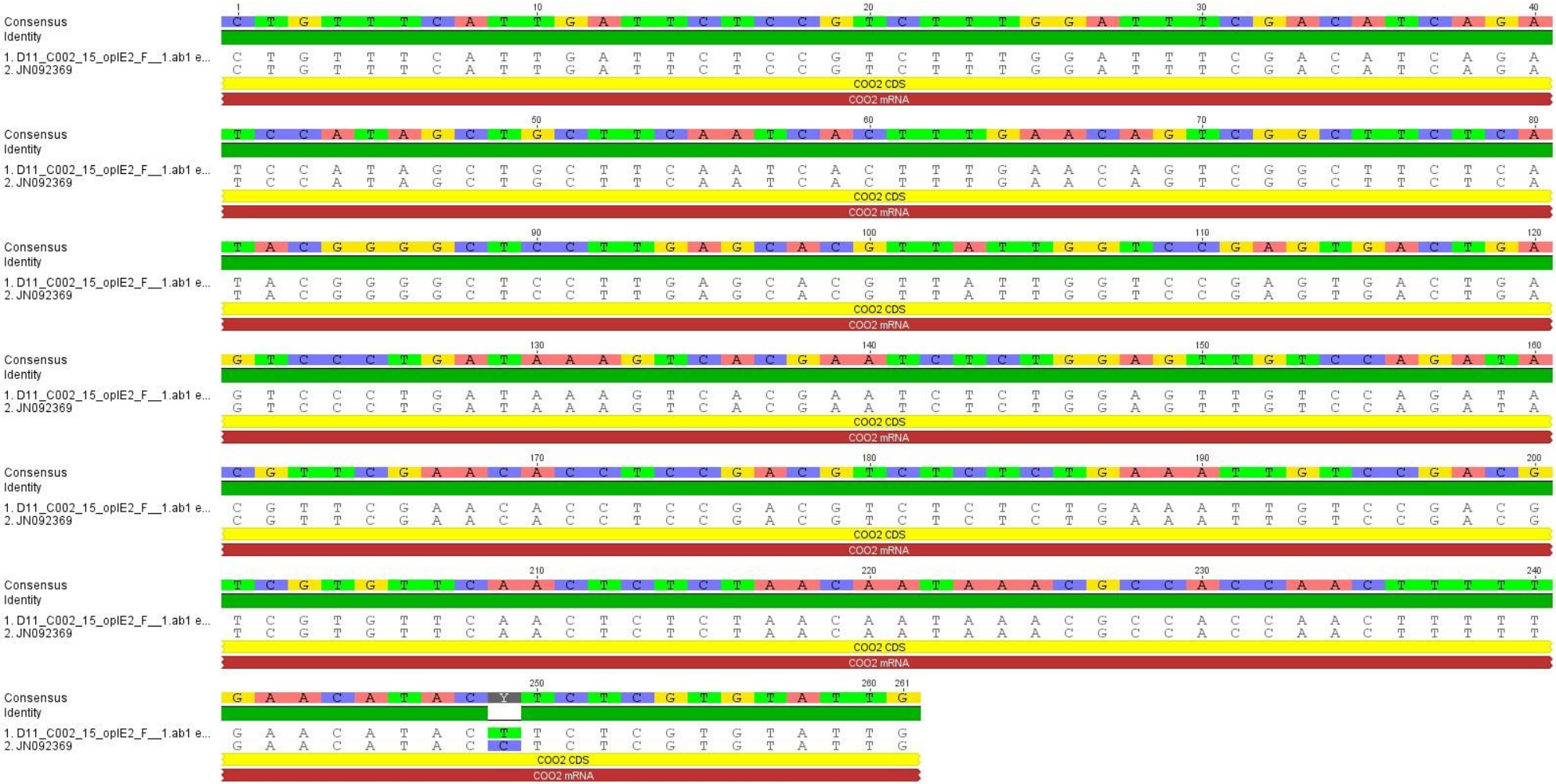


Figure 4.5 (a): Sequence alignment of *C002* cloning data. The sequencing data obtained from cloning *C002* into the pIZT/V5-His vector indicates significant alignment with *Diuraphis noxia C002 gene* [GenBank: JN092369.1 (Figure A1)].

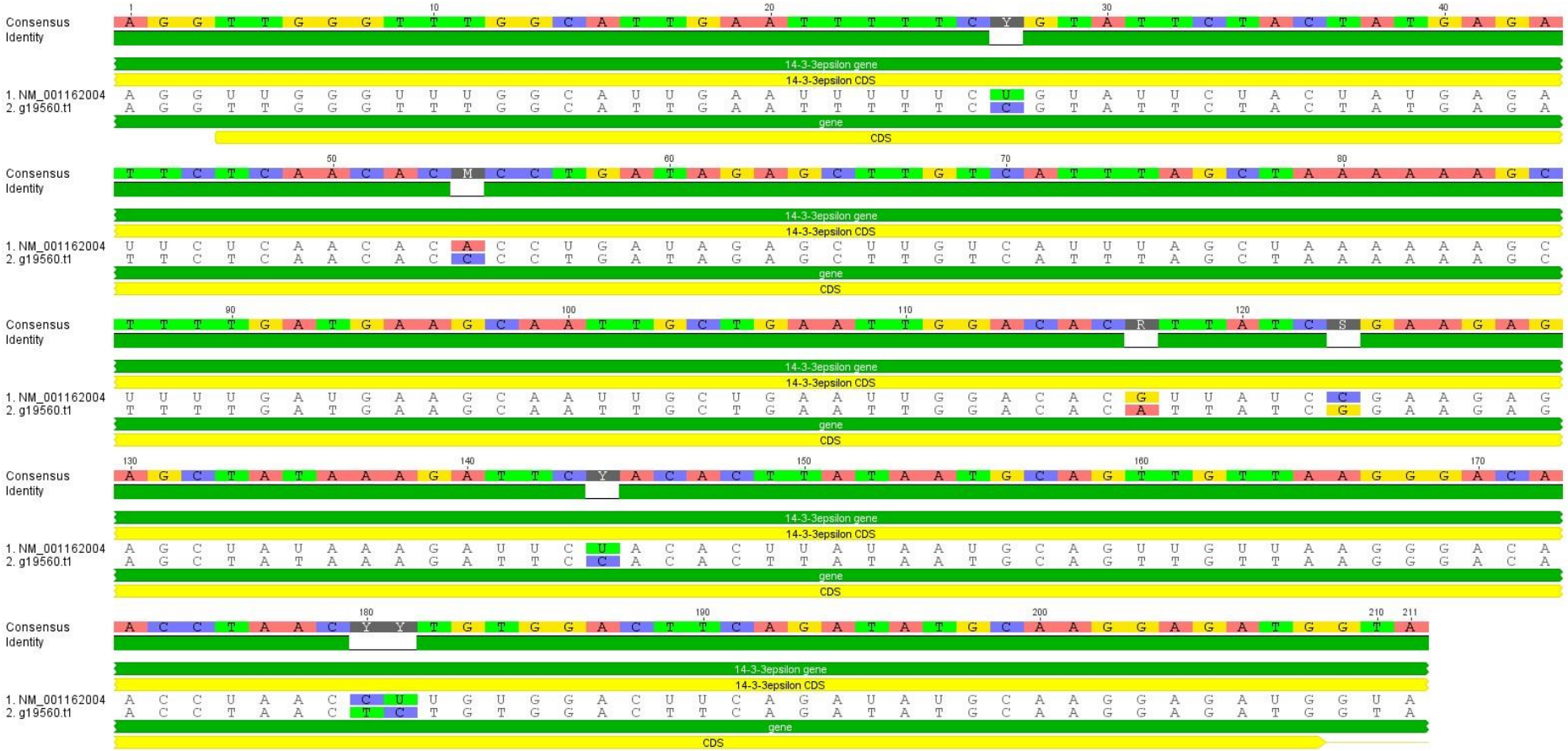


Figure 4.5 (b): Sequence alignment between *Acyrthosiphon pisum 14-3-3 protein epsilon* [NCBI Reference Sequence: NM_001162004.2 (Figure A2)] mRNA sequence to that from RWAs genome. Scaffold g19560.t1 used for subsequent primer- and siRNA designing.

4.3.*2* Relative expression of *C002* and *14-3-3 ε* in SAM vs. SA1 at 0h

Relative expression of *C002* and *14-3-3 ε* in SAM vs. SA1 at 0 hours was determined using *L27* and *L32* as reference genes. When compared to SA1, SAM has a 3.45 and 2.92 fold higher relative expression of *C002* when utilising *L27* and *L32* as reference genes respectively, while *14-3-3 ε* displays a 2.63 and 2.23 fold higher relative expression in SAM (Table A1; Figure 4.6). The results from the student t-test indicated that the differences in the relative expression values of both transcripts in SA1 compared to SAM are indeed statistically significant as the p-values were all smaller than 0.05 (p≤ 0.05) (Table A2).

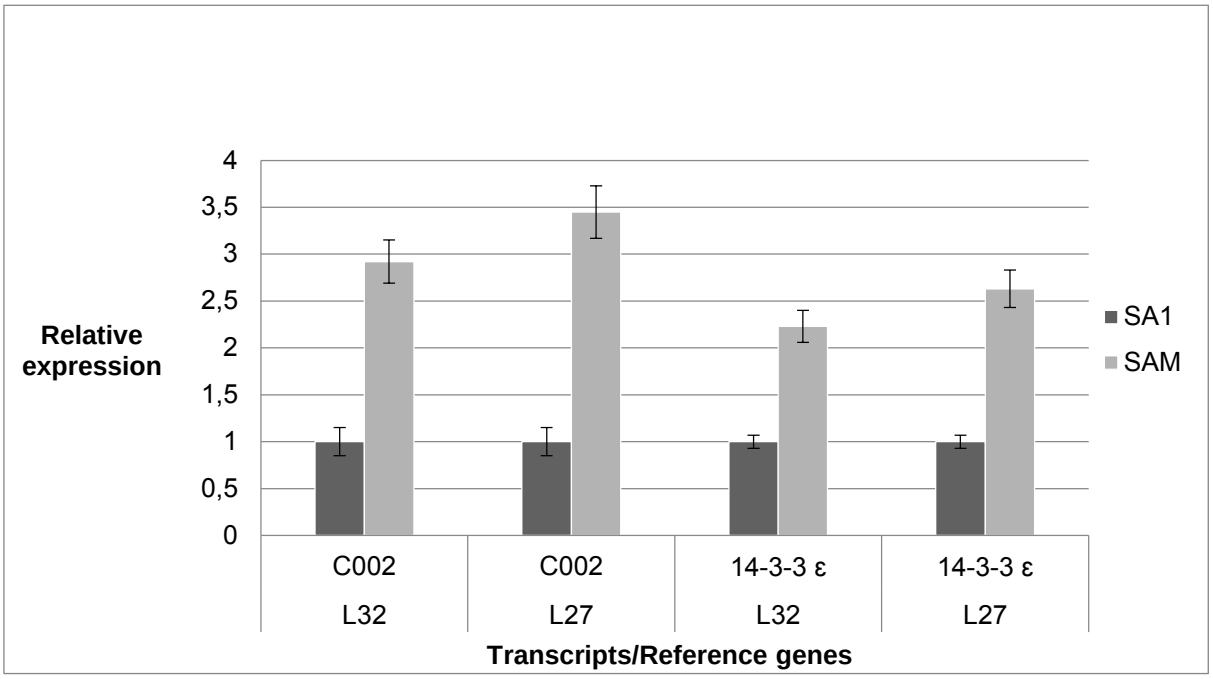


Figure 4.6: Relative expression of *C002* and *14-3-3 ε* in SAM vs. SA1 at 0h using *L27* and *L32* as reference genes. Both *C002* and *14-3-3 ε* display higher relative expression in SAM (light grey) when compared to SA1 (dark grey).

4.3.3. Relative expression and fecundity data of *C002* at different time points in control aphids vs. aphids exposed to *C002* siRNA

4.3.3.1 Feeding on artificial medium containing *C002* siRNA

According to the RT-qPCR results, it seems as though *C002* silencing takes effect at 6h already [Figure 4.7 (b)] as a higher level of expression is observed in the control aphids

when compared to the suppressed *C002* expression level in the aphids subjected to *C002* siRNA when added to artificial medium. However, a more prominent difference in expression levels between the two groups of aphids is seen at 24h after treatment exposure [Figure 4.7 (a) and (b)]. At the 24h time-point, the relative expression of *C002* is 144.72 and 3.23 (*L27* and *L32* respectively) in the control aphids compared to 15.05 and 1.68 (*L27* and *L32* respectively) in the siRNA exposed aphids (Table A3). P-values obtained from student t-tests completed on SigmaPlot®, verify the statistical significance of the observed differences in relative expression at 24h between control and siRNA exposed aphids as both p-values are smaller than 0.05 ($p \leq 0.05$) (Table A4). This indicates that silencing of *C002* occurred in the siRNA exposed aphids and thus that the RNAi treatment was successful. The relative expression levels seem to be higher in the siRNA exposed aphids vs. the control aphids at 6h (for *L27* only), 48h and 72h (Figure 4.7), however the p-values as results of the student t-tests shows that the differences detected are not statistically significant as these values are not smaller than 0.05 (except at 72h for when *L27* are utilised) (Table A4). Therefore, the expression levels of *C002* appear to be similar in the control and siRNA exposed aphids at 48h and 72h indicating that the transient silencing effect has subsided, with expression levels returning to normal.

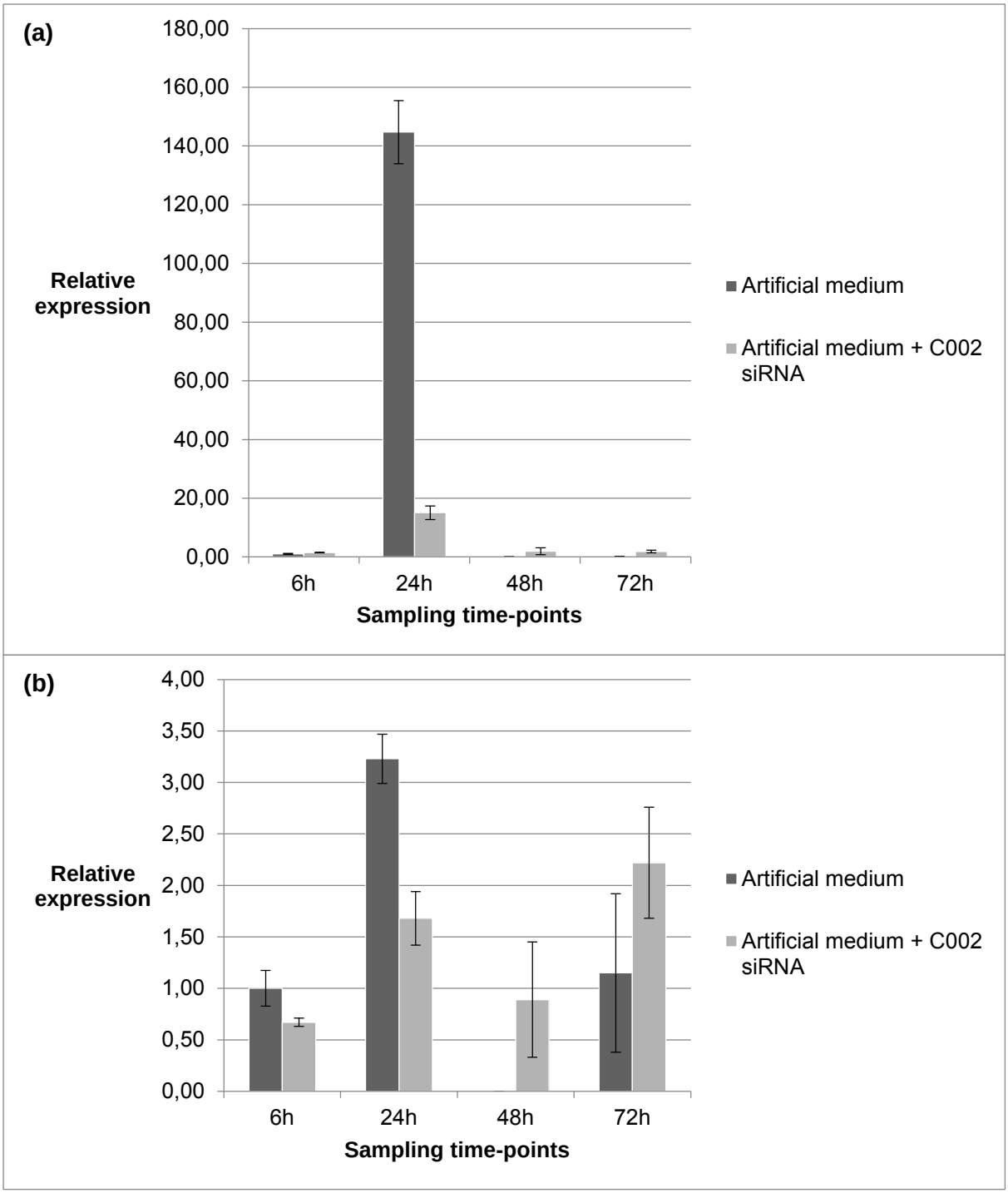


Figure 4.7: Relative expression of *C002* in control aphids feeding on artificial medium (dark grey) compared to aphids feeding on artificial medium containing *C002* siRNA (light grey) at different time points, with *L27* **(a)** and *L32* **(b)** utilised as reference genes (SE values also indicated).

The number of nymphs produced by the control aphids and the *C002* siRNA exposed aphids were counted at 0h, 24h, 48h and 72h and the total nymph production of the six control aphids and the six *C002* siRNA exposed aphids (Table A7) was calculated at the end of the trial (Figure 4.8). For both the control and *C002* siRNA exposed aphids, only two of the six adult aphids gave birth to young (Table A7), while the latter produced more nymphs overall when compared to the control aphids.

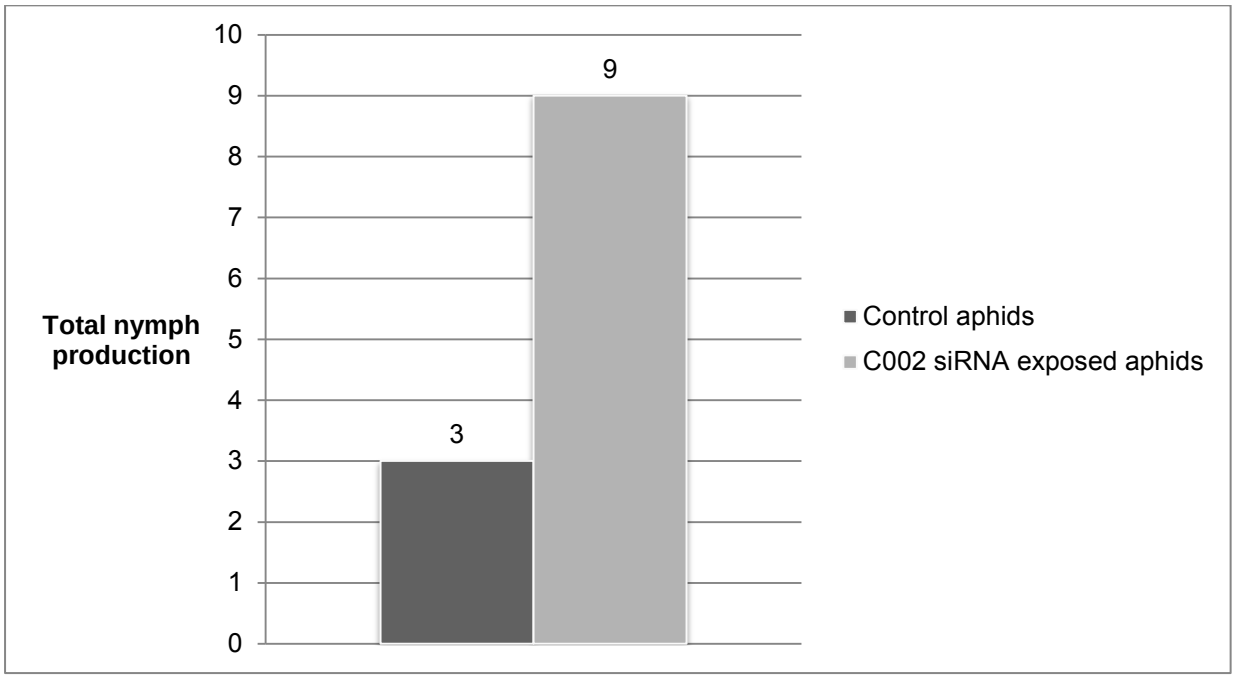


Figure 4.8: Fecundity data of aphids after exposure to *C002* siRNA. Where control aphids (dark grey) feeding on artificial medium are compared to aphids feeding on artificial medium containing *C002* siRNA (light grey) at different time points. Only 33% of the adult female RWAs produced nymphs.

4.3.3.2 Feeding on *C002* siRNA injected wheat leaves

The results obtained from RT-qPCR analysis indicates higher expression of *C002* in the control aphids compared to the *C002* siRNA exposed aphids at 6h, 24h, and 48h [Figure 4.9 (a) and (b)], as higher levels of this transcript is observed in the control aphids. At 72h, the relative expression level of *C002* is observed to be lower in the control aphids compared to the aphids subjected to *C002* siRNA (Figure 4.9; Table A5). These findings are visible when utilising both *L27* and *L32* as reference genes. However, the standard deviations for the control aphids at 6h and 24h and for the *C002* siRNA exposed aphids at 24h and 72h (for *L27* and *L32*) are higher than expected indicating that the individual responses of the aphids within these groups appear to fluctuate a great deal. It is only at 48h that the standard deviation is satisfactory, therefore suggesting that the effect of *C002* silencing is most eminent at this time-point. The student t-test results also show that none of the differences in expression levels observed at any time-point was statistically significant ($p \geq 0.05$), except for after 48h of feeding when utilising *L32* (Table A6). Thus, it seems that silencing is initiated at 6h post exposure already and continues until 48h, whereafter the expression levels of *C002* normalise again.

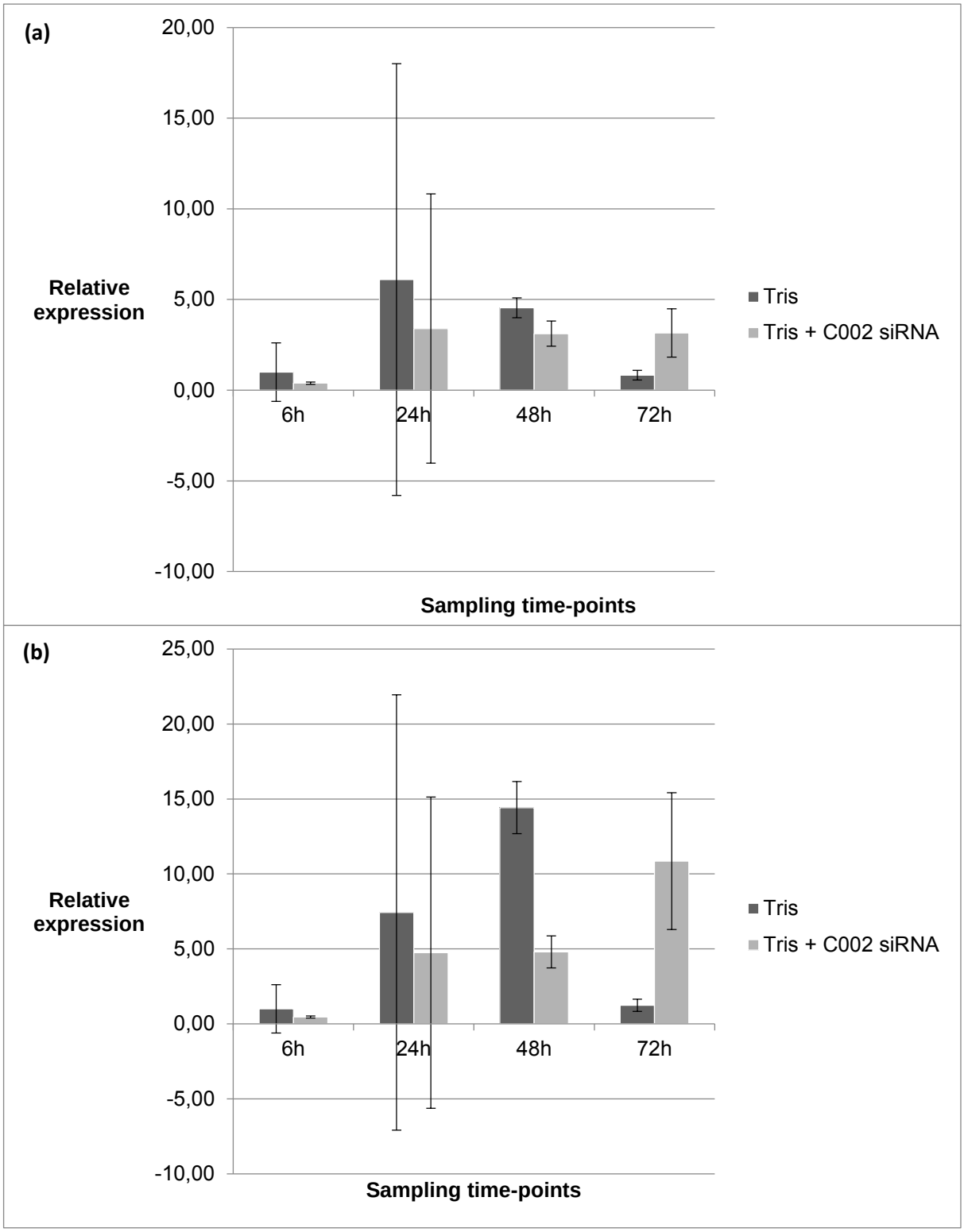


Figure 4.9: Relative expression of *C002* at different time points in control aphids feeding on Tris injected wheat leaves (dark grey) compared to aphids feeding on wheat leaves injected with Tris and *C002* siRNA (light grey), with *L27* **(a)** and *L32* **(b)** utilised as reference genes (SE values also indicated).

Again the number of nymphs produced by the control aphids and the *C002* siRNA exposed aphids were counted at 0h, 24h, 48h and 72h and the total nymph production of the six control aphids and the six *C002* siRNA exposed aphids (Table A7) was calculated at the end of the trial (Figure 4.10). Of the six control aphids, four managed to produce nymphs while only half of the *C002* siRNA exposed aphids gave birth to nymphs (Table A7). Overall, the control aphids produced more nymphs when compared to the *C002* siRNA exposed aphids (Figure 4.10).

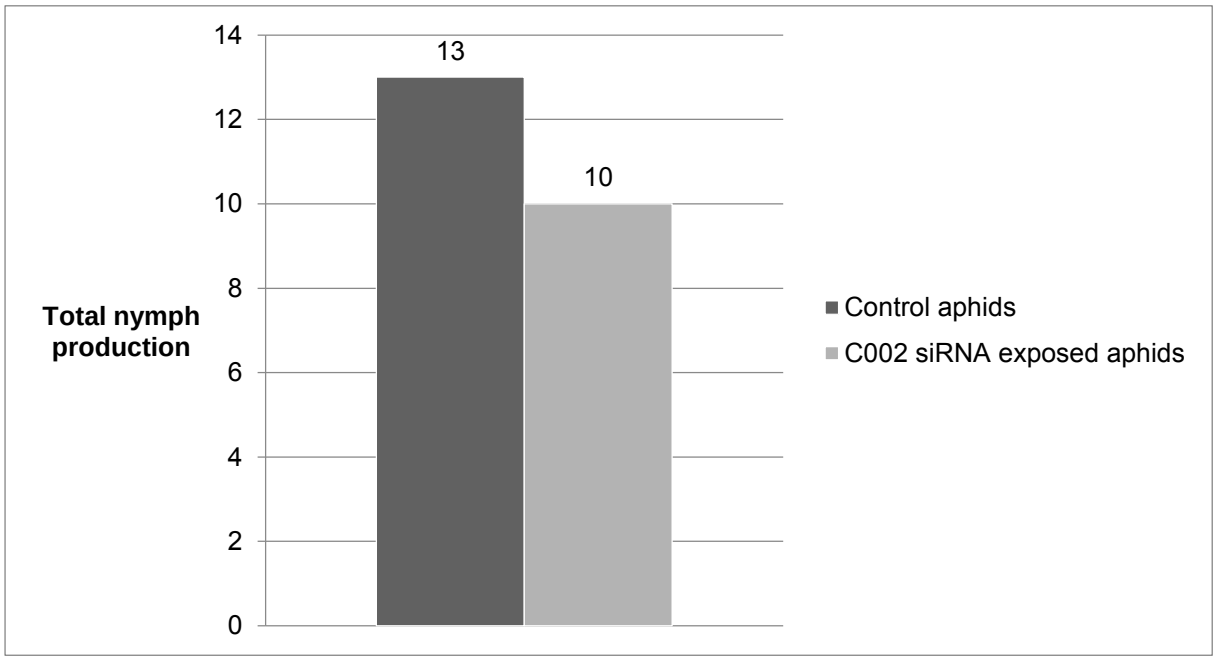


Figure 4.10: Fecundity data of aphids after exposure to *C002* siRNA. Where control aphids (dark grey) feeding on Tris injected wheat leaves are compared to aphids feeding on wheat leaves injected with Tris containing *C002* siRNA (light grey) at different time points. Only 66% and 50% of the control and siRNA-exposed adult female RWAs, respectively, produced nymphs.

4.3.4. Relative expression and fecundity data of *14-3-3 ε* at different time points in control aphids vs. aphids exposed to *14-3-3 ε* siRNA

4.3.4.1 Feeding on artificial medium containing *14-3-3 ε* siRNA

The RT-qPCR results show higher levels of *14-3-3 ε* expression at 24h after initial siRNA exposure in the control aphids when compared to the aphids that fed on artificial medium holding *14-3-3 ε* siRNA [Figure 4.11 (a) and (b)]. The relative expression of *14-3-3 ε* at this time-point in the control aphids is 9.95 and 27.44 (*L27* and *L32* respectively), while the *14-3-3 ε* siRNA exposed aphids display relative expression of 2.99 and 2.71 (*L27* and *L32* respectively) (Table A8). Despite the large SE values, it is evident from the student t-test results that the differences observed in the relative expression between the control and *14-3-3 ε* siRNA exposed at 24h is statistically significant because the p-values are smaller than 0.05 ($p \leq 0.05$) (Table A9). Therefore, it can be concluded that successful silencing, and thus RNAi, of *14-3-3 ε* occurred at 24h in the siRNA exposed aphids. Aphids subjected to *14-3-3 ε* siRNA appear to have higher relative expression of this transcript at 6h, 48h, and 72h when compared with control aphids (Figure 4.11), but the student t-tests demonstrates that these differences are not statistically significant (Table A9). Similar to *C002*, it appears that the expression levels normalise after 24h as the transient silencing effect tapers off.

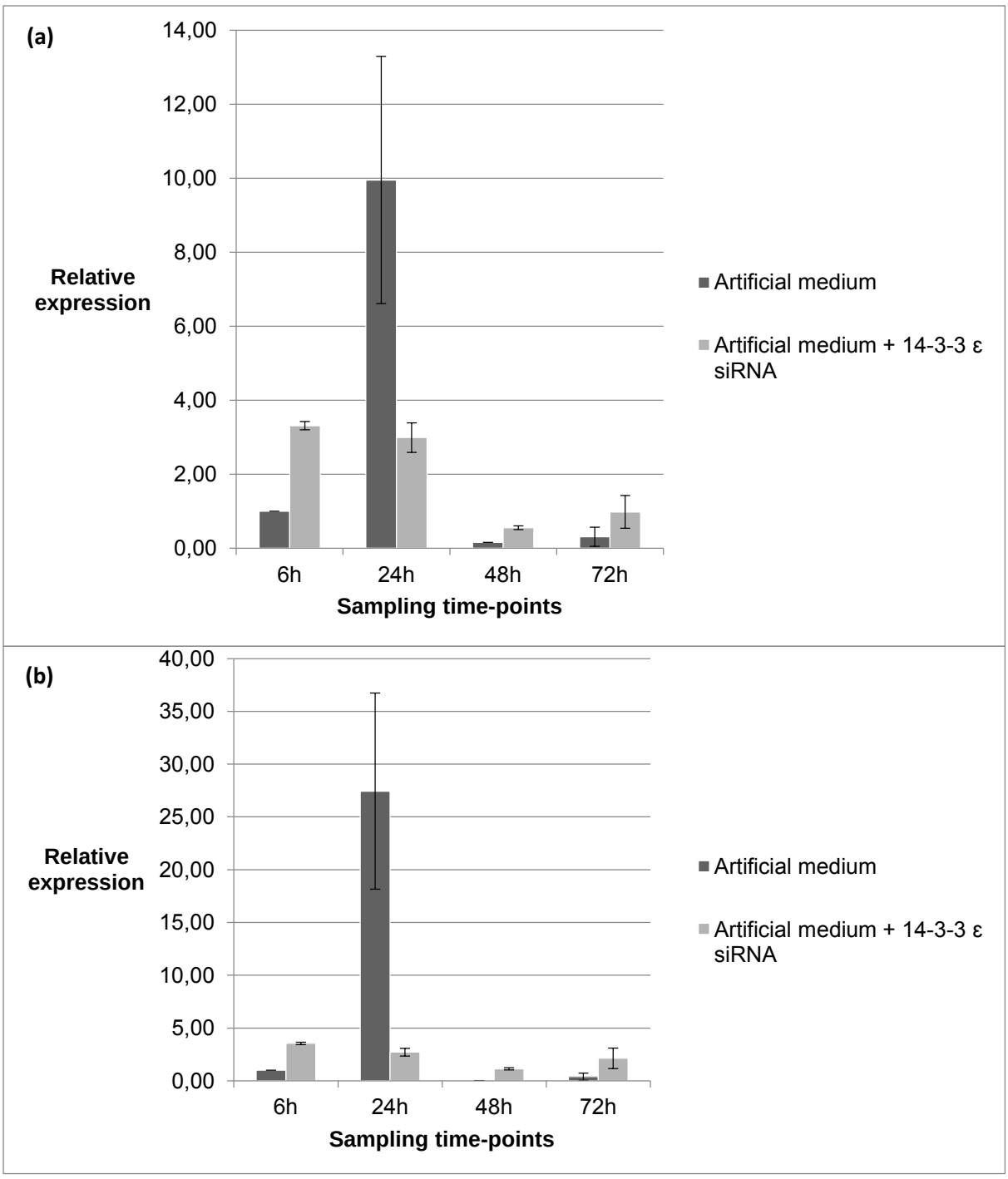


Figure 4.11: Relative expression of *14-3-3 ε*, at different time points, in control aphids feeding on artificial medium (dark grey) compared to aphids feeding on artificial medium containing *14-3-3 ε* siRNA (light grey), with *L27* **(a)** and *L32* **(b)** utilised as reference genes (SE values also indicated).

Similarly to the *C002* trials, the number of nymphs produced by the control aphids and the *14-3-3 ε* siRNA exposed aphids were counted at 0h, 24h, 48h and 72h and the total nymph production of the six control aphids and the six *14-3-3 ε* siRNA exposed aphids (Table A7) was calculated at the end of the trial (Figure 4.12). While only two of the six control aphids produced nymphs, all but one of the aphids exposed to *14-3-3 ε* siRNA gave birth to young (Table A7). The total amount of nymphs produced by the *14-3-3 ε* siRNA exposed aphids were significantly higher in comparison to the control aphids.

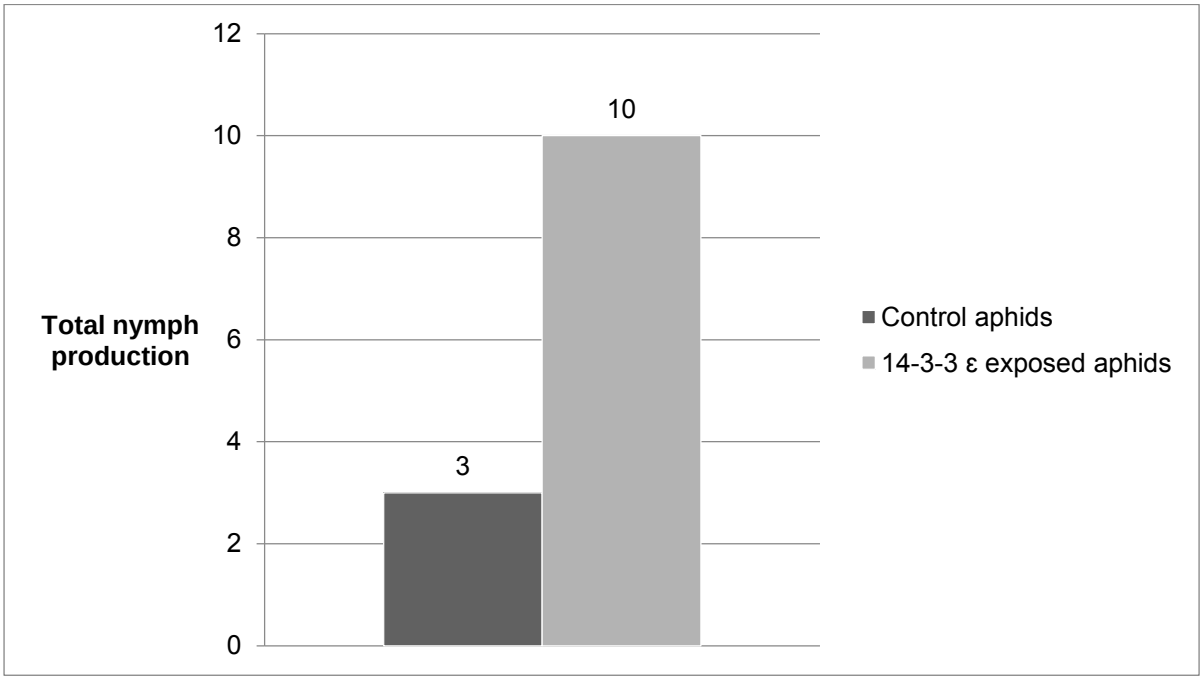


Figure 4.12: Fecundity data of aphids after exposure to *14-3-3 ε* siRNA. Where control aphids (dark grey) feeding on artificial medium are compared to aphids feeding artificial medium containing *14-3-3 ε* siRNA (light grey) at different time points. Only 33% and 84% of the control and siRNA-exposed adult female RWAs, respectively, produced nymphs.

4.3.4.2 Feeding on *14-3-3 ε* siRNA injected wheat leaves

Higher levels of *14-3-3 ε* relative expression is observed at 24h in the control aphids when compared to the aphids subjected to *14-3-3 ε* siRNA [Figure 4.13 (a) and (b)], while a slight increase is observed at 6h when using *L32*. The relative expression of this transcript at 24h

is 8.31 and 4.72 in the control aphids vs. 1.53 and 1.08 in the test aphids when using *L32* and *L27* respectively (Table A10). Nonetheless, these differences were not found to be statistically significant after performing student t-test on SigmaPlot® (Table A11) as none of the p-values were smaller than 0.05. At 6h (only *L27*), 48h and 72h higher levels of relative expression are seen in the *14-3-3 ε* siRNA exposed aphids when compared to the control aphids (Figure 4.13), but again these differences have not proven to be statistically significant (Table A11). Almost all of the standard deviations observed at the different time-points are higher than expected, therefore indicating that the responses of the individual aphids within each group appear to differ greatly. Thus, it appears that transient silencing takes place but due to the method used the differences in expression levels are not statistically significant.

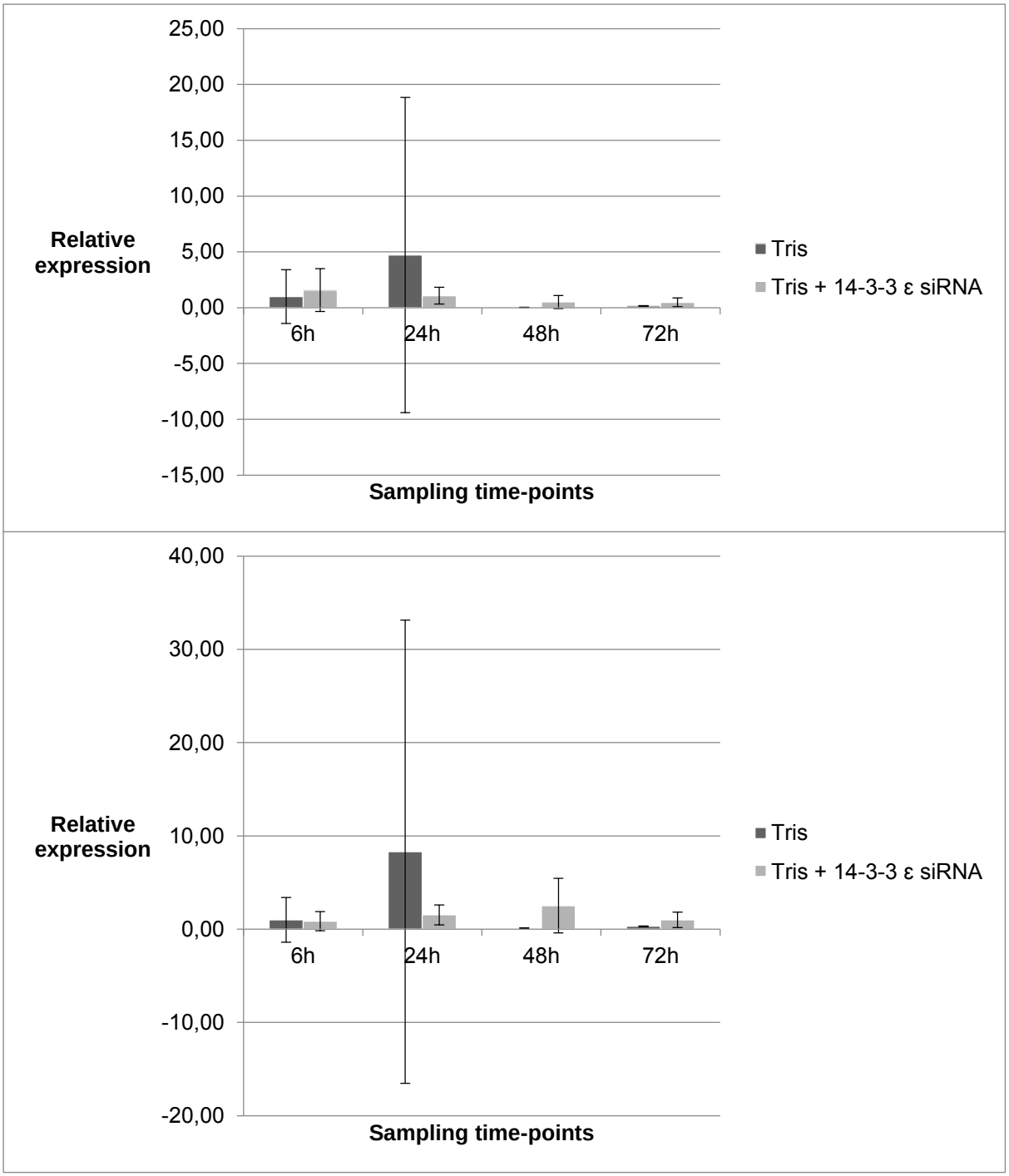


Figure 4.13: Relative expression of *14-3-3 ε* at different time points in control aphids feeding on Tris injected wheat leaves (dark grey) compared to aphids feeding on wheat leaves injected with Tris and *14-3-3 ε* siRNA (light grey), with *L27* **(a)** and *L32* **(b)** utilised as reference genes (SE values also indicated).

During the leaf injection trial, the number of nymphs produced by the control aphids and the *14-3-3 ε* siRNA exposed aphids were also counted at 0h, 24h, 48h and 72h and the total nymph production of the six control aphids and the six *14-3-3 ε* siRNA exposed aphids (Table A7) was calculated at the end of the trial (Figure 4.14). Four of the six control aphids managed to produced nymphs, while all of the aphids exposed to *14-3-3 ε* siRNA gave birth to young (Table A7). The total amount of nymphs produced by the *14-3-3 ε* siRNA exposed aphids was higher when compared to the control aphids (Figure 4.14).

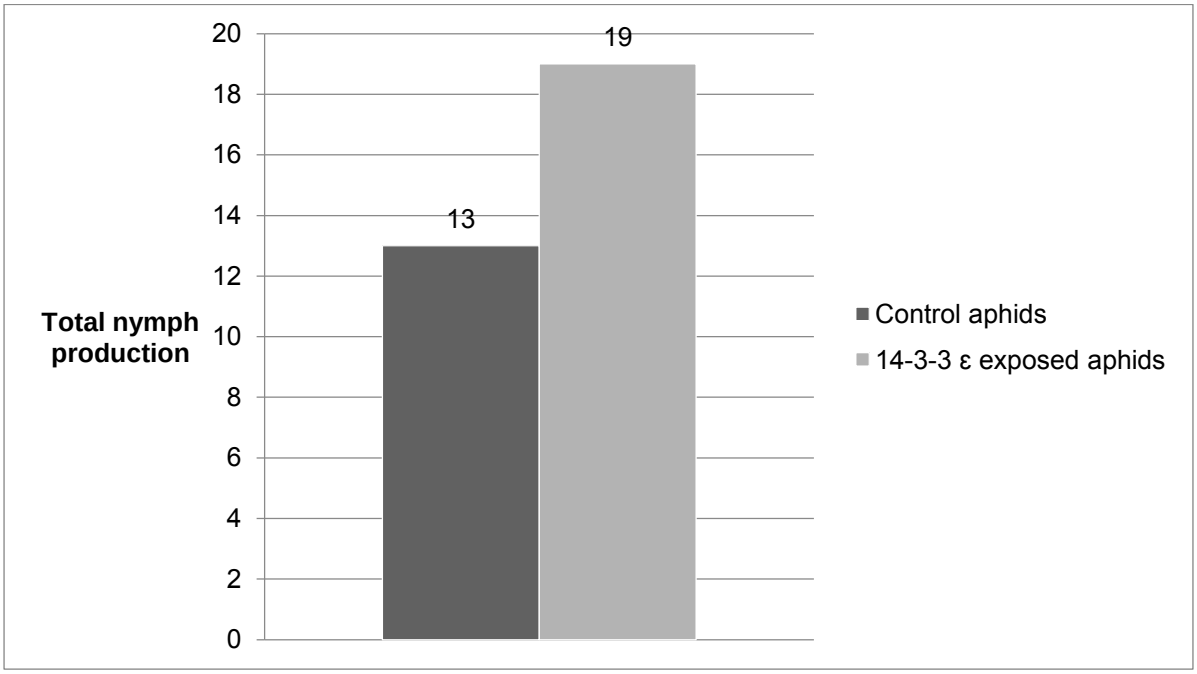


Figure 4.14: Fecundity data of aphids after exposure to *14-3-3 ε* siRNA. Where control aphids (dark grey) feeding on wheat leaves injected with Tris are compared to aphids feeding on wheat leaves injected with Tris containing *14-3-3 ε* siRNA (light grey) at different time points. Of the adult female RWAs, 66% and 100% of the control and siRNA-exposed aphids, respectively, produced nymphs.

4.4 Discussion

It is becoming clearer that plant feeding insects' salivary repertoires are just as complex as the broadly studied saliva of blood feeding insects (Rao *et al*. 2013). Some of the first studies done with saliva collections revealed enzymatic activities in aphid saliva, therefore suggesting the presence of different types of enzymes, such as oxidoreductases and hydrolases (Leszczynski & Dixon 1990; Ma *et al*. 1990; Miles & Oertli 1993). The accessibility of advanced proteomic tools, combined with the available aphid genome (http://www.cg-base.org) and transcriptome sequences, opened the opportunity for direct identification of aphid salivary proteins (Rodriguez & Bos 2013; Jaouannet *et al*. 2014) (Table 4.7). Studies exploring this avenue identified (predicted) several secreted salivary proteins, or candidate effectors. Some of these proteins have predicted activities, including cell-wall degrading enzymes (such as pectinases, glucanases, and amylases) and detoxifying enzymes (e.g. oxidoreductases, phenol oxidases, and peroxidases) (Carolan *et al*. 2009; Cooper *et al*. 2010, 2011; Nicholson *et al*. 2012; Rao *et al*. 2013). The bulk of the candidate effectors, however show no similarity to proteins of predicted function, some of which are unique to aphids (Rodriguez & Bos 2013). As seen in Table 4.7, only a small number of the identified effector proteins have been characterised until now. Results indicate that these effector proteins are involved in promoting or decreasing virulence and activating or suppressing defences (Rodriguez & Bos 2013). In a study conducted by Nicholson *et al*. (2012), it was found that the protein composition of RWA saliva differs from other aphids, therefore proposing that aphids form unique associations with their hosts. It was also observed that specific proteins uniquely expressed in the RWA salivary proteome, differ between the different biotypes (Nicholson *et al*. 2012). Four RWA salivary secretion proteins have previously been identified by Cloete (2015), which was set out to be investigated in the present study. These included *C002*, *14-3-3 epsilon (ε)* protein, LOC100169243 uncharacterised protein, and apolipophorins protein.

Table 4.7: Summary of characterised aphid effector proteins (Jaouannet *et al*. 2014).

Effector	Aphid species	Role	Molecular activity	Reference
Mp55	*Myzus persicae*	Suppression of plant defences	Lower accumulation of 4-methozyindol-3-ylmethylglucosinolate, callose and H_2O_2	Elzinga & Jander (2013)
C002	*Acyrthosiphon pisum; Myzus persicae*	Essential for aphid feeding	So far unknown	Mutti *et al*. (2006, 2008); Bos *et al*. (2010); Pitino *et al*. (2011)
Mp 1/PIntO1	*Myzus persicae*	Enhanced aphid fecundity	So far unknown	Bos *et al*. (2010); Pitino & Hogenhout (2013)
PIntO2	*Myzus persicae*	Enhanced aphid fecundity	So far unknown	Pitino & Hogenhout (2013)
Mp10	*Myzus persicae*	Reduced aphid fecundity	Altering JA- and SA-defense related signalling in plants	Bos *et al*. (2010); Rodriguez *et al*. (2014)
Mp42	*Myzus persicae*	Reduced aphid fecundity	Perturbation f nuclear envelope and membranes; aggregate formation in ER	Bos *et al*. (2010); Rodriguez *et al*. (2014)
Me23	*Macrosiphon eurphirbiae*	Enhanced aphid fecundity	So far unknown	Atamian *et al*. (2013)
Me10	*Macrosiphon eurphirbiae*	Enhanced aphid fecundity	So far unknown	Atamian *et al*. (2013)

The LOC100169243 uncharacterised protein was included in the current study to investigate its activity during the RWA-wheat interaction. Unfortunately difficulties during the cloning process led to the omission of the transcript from the study due to time-constraints, but should be explored in future work.

In a study conducted by Carolan *et al*. (2011), two lipid-binding apolipophorins were identified in the predicted secreted salivary proteins. It was suggested that these proteins undergo a conformational change after binding to lipid elicitor molecules, which can induce the insect innate immune response (Whitten *et al*. 2004). Therefore, it is possible that the apolipophorins proteins might interfere with the plant's own cellular immune response signalling (Carolan *et al*. 2011). However, further studies, such as the present one, is still needed to confirm these findings. During the analysis of the sequencing data obtained from cloning, it appeared that no homolog of this protein is present in the RWA's genome. This finding led to the exclusion of apolipophorins protein from further experimental procedures and analysis in the study.

C002 is a highly abundant salivary protein with unknown function, first identified in *A. pisum* and is delivered inside the host plant tissue during feeding (Mutti *et al*. 2008). It appears to be primarily expressed in the salivary glands of pea aphids (*Acyrosiphon pisum*) and green peach aphids (*Myzus persicae*) (Yong *et al*. 2014). However, more recently semi-quantitative PCR analysis indicated low expression of *C002* in the guts of *A. pisum* (Mutti *et al*. 2006; Pitino *et al*. 2011). Furthermore, Mutti *et al*. (2008) reported on the presence of C002 protein in host plant fava beans post *A. pisum* feeding. This proposes that aphid feeding is accompanied by the secretion of C002 protein into the host plant. In the current study the presence of *C002* in the RWA was verified via cloning and sequencing and the RT-qPCR results indicated that this transcript has a higher relative expression in SAM, the most virulent RWA biotype, compared to SA1, the least virulent RWA biotype in South Africa (Figure 4.6). The relative expression differences were proven to be statistically significant using SigmaPlot® (Table A2). Thus, proposing *C002's* potential role as effector protein

conferring virulence to the RWA. The saliva of *A. pisum* and *M. persicae* have been shown to contain C002 protein (Harmel *et al*. 2008; Carolan *et al*. 2009), whereas phylogenetic analysis indicated that this gene is fast-evolving in aphids, while it is not found in other insects (Ollivier *et al*. 2010). Research pertaining to *C002* has mainly been conducted on *M. persicae* and *A. pisum*. Thus, taking into account all above mentioned results together with early results obtained in this study, *C002* was included in the feeding with artificial diet and injection of siRNA into wheat plants trials to investigate its role in RWA-wheat interaction.

Expression analysis was performed via RT-qPCR, using the sampled aphids from both experimental trials. From the results of the feeding with artificial diet in a tube experiment, it was evident that silencing of *C002* was achieved through RNAi. At 6h (when utilising *L32* only) and 24h (*L27* and *L32*) post exposure to *C002* siRNA, the aphids subjected to *C002* siRNA displayed lower relative expression levels of this transcript in contrast to control aphids (Figure 4.7; Table A3). The t-test results (Table A4) verified that the observed expression differences were only statistically significant at 24h (p-values smaller than 0.05). At 6h (*L27*), 48h and 72h after initial siRNA treatment the levels of *C002* expression are higher in the *C002* siRNA exposed aphids compared to the control aphids. The differences in relative expression levels at aforementioned time-points, however, were not found to be statistically significant (p-values larger than 0.05). Thus, it can be proposed that between 6h and 24h silencing of *C002* occurs and thereafter similar expression levels are observed between the control and aphids subjected to siRNA as the transient silencing effect wears off. Comparable results are produced from the RT-qPCR analysis performed on aphids who were allowed to feed on wheat leaves injected with *C002* siRNA. When utilising both *L27* and *L32* as reference genes, it seems that silencing is visible at 6-48h after initial exposure to *C002* siRNA (Figure 4.9; Table A5). However, the standard deviations at 6h and 24h are not ideal and indicate that the expression of this transcript differs a great deal between the aphids in each group. For this experiment 1 µl of 1µg/µl siRNA dissolved in 10 mM Tris was injected into the bottom vein of the wheat leaf using a Hamilton syringe and 1 mm needle.

During the injection, it is possible that the total volume of siRNA did not enter the vein at some of the injection sites, therefore resulting in different concentrations of siRNA available when aphid feeding commences. As aphids used for RT-qPCR analysis were sampled from different injection sites, the aforementioned could explain the variations observed in *C002* expression levels between aphids within the same group (time-point) - especially at 6h and 24h. T-tests were performed and it was found that none of the observed expression level differences are statistically significant with p-values larger than 0.05 (Table A6). Thus, it seems that the artificial diet experimental setup is a more accurate method for investigating the silencing of *C002*, because the aphids are inevitably subjected to the same concentration of siRNA. Through the injection of siRNA molecules into the abdomen of pea aphids, Mutti *et al*. (2006; 2008) demonstrated that the *C002* protein fulfils a vital role in some aspects of the foraging and feeding behaviour of aphids. Knockdown of this salivary transcript caused the pre-mature death of *A. pisum*, possibly attributable to the aphids being in contact with the phloem sap for a short amount of time as a result of modification in its feeding behaviour (Mutti *et al*. 2006; 2008). In another study (Bos *et al*. 2010), the overexpression of *C002* in *Nicotiana benthamiana* improved the reproduction rate of *M. persicae* significantly, therefore highlighting its role in aphid virulence. A significant drop in reproduction rate, but not survival rate, has also been observed due to the knockdown of *MpC002* via the feeding of *M. persicae* on *N. benthamiana* leaf discs as well as on *A. thaliana* plants engineered to produce dsRNAs targeted against *C002* (Pitino *et al*. 2011). During both trials, the fecundity of adult RWAs were monitored by counting the number of nymphs present at 24h, 48h and 72h. This was done to determine the effect of the RNAi on the survival and reproduction of the RWAs. When comparing the total nymph production, somewhat opposing results are observed. It seems that the suppressed levels of *C002* brought about by the RNAi during the leaf injection trial had an effect on the reproduction of the aphids as control aphids produced more nymphs than the siRNA exposed aphids (Figure 4.10), thus corroborating the findings of Pitino *et al*. (2011). However, the inverse is seen in the fecundity data obtained during the artificial medium trial with more nymphs being

produced by the siRNA subjected aphids (Figure 4.8). Only 2 and 3 aphids (feeding on artificial medum holding *C002* siRNA and feeding on wheat leaves injected with *C002* siRNA, respectively) gave birth to nymphs (Table A7), probably as a result of the *C002* siRNA exposure and the adult RWAs thus being more hesitant to subject their young to these conditions. Adult aphids were found dead at 48h and 72h for the injection trial (Table A7) and this could either be due to aphids getting stuck in the bit of glue exposed in the aphid cage or as a result of successful silencing impairing the survival of the adults. The proposed silencing of *C002* detected at 6-24h could pose a possible explanation for the decrease in survival of aphids seen at 48-72h in the fecundity data. However, the custom-made aphid cages (Figure 4.3) used to restrict feeding at the siRNA injection site appears to be inapt as the aphids can get stuck in exposed glue of the double-sided tape where the hole is punched and this might skew the results. Therefore, a definitive and accurate conclusion cannot be drawn with regards to the effect of *C002* silencing on the survival and reproduction of the RWA and alternative containment options need to be investigated and tested.

The 14-3-3 epsilon (ε) protein is part of a family of highly homologous proteins, which has been described in all eukaryotic organisms ranging from yeast (Van Heusden *et al*. 1995) and *Drosophila* (Skoulakis & Davis 1996) to *Xenopus* (Martens *et al*. 1992) and humans (Boston & Jackson 1980). The 14-3-3 proteins are known to modulate interactions between proteins and play a role in cell signalling, regulation of cell cycle progression, intracellular targeting/trafficking, cytoskeletal structure, and transcription (Aitken 2006). This class of proteins have the ability to interact with more than 200 target proteins through phosphoserine-dependent and phosphoserine-independent approaches (Mhawech 2005). However, little is known regarding the consequences of these interactions and is therefore the subject of continuing studies. Cloete (2015) suggested that 14-3-3 ε protein might potentially be a protein elicitor present in aphid saliva, but further research needs to be conducted to investigate this notion. Nonetheless, the function of this protein effector in

aphid, or RWA virulence is still uncertain and therefore it is included in the present study. Thus, *14-3-3 ε* formed part of this study to determine its role in the interaction between RWA and its host plant wheat. The same as with *C002*, RT-qPCR analysis showed higher relative expression levels of this transcript in the most virulent RWA biotype, SAM, when compared to the least virulent biotype, SA1 (Figure 4.6). These results therefore suggest that *14-3-3 ε* can possibly be seen as an effector protein adding to the virulence of the RWA. RT-qPCR results pertaining to the feeding with artificial diet trial were similar to those observed for *C002*. At 24h the control aphids displayed higher relative expression in contrast to the aphids exposed to *14-3-3 ε* siRNA (Figure 4.11). The statistical significance of the detected expression differences was confirmed via a student t-test (Table A9). In contrast to *C002*, the higher levels of *14-3-3 ε* in control aphids are not observed at 6h or 48h. The t-tests also demonstrated that the differences in relative expression levels seen at 6h, 48h, and 72h were not statistically significant as p-values were lower than 0.05 (Table A9). Therefore, it can be theorised that successful silencing occurred, and thus RNAi, of *14-3-3 ε* at 24h in control aphid in comparison to siRNA exposed aphids. Analysis of the results obtained for the RT-qPCR performed on aphids subjected to feeding on *14-3-3 ε* injected wheat leaves displayed comparable trends to the results of the artificial diet trials. Again, this transcript displayed higher levels of expression in control aphids at 24h (also at 6h when utilising *L32*) only, while the inverse in visible at 6h (*L27*), 48h and 72h after initial exposure to siRNA (Figure 4.13). However, as with the *C002* the standard deviations observed at 6-48h are quite high, indicating that a lot of variation in the level of *14-3-3 ε* expression of individual aphids within the same group is picked up. Also, none of the differences were proven to be statistically significant after performing student t-tests (Table A11). Therefore, highlighting once more the lack in uniformity and accuracy of the siRNA injection method in comparison to using the artificial diet setup. For the *14-3-3 ε* siRNA treatment, all but 1 adult aphid produced nymphs (adult RWA 2 of feeding trial) as observed in the fecundity data (Table A7). The total nymph production is signicantly lower in the control aphids during the artificial

medium trial (Figure 4.12), while this group of aphids were also less prone to giving birth to nymphs compared to siRNA exposed aphids during the leaf injection experiment (Figure 4.14). The aforementioned results are quite surprising as it would be expected to observe the inverse when taking into account the RT-qPCR results. RNAi caused by *14-3-3 ε* siRNA might possibly be more slow acting than that of *C002* siRNA, as more adults exposed to the former gave birth to nymphs (also from earlier time points) and decreased levels of *C002* in the siRNA exposed aphids are seen as early as 6h after feeding is initiated. Additionally, dead aphids were only observed at 72h post incubation for treatment aphids compared to *C002* treatment aphids having dead aphids at 48h already. However, due to previously mentioned inadequacies and time constraints the ultimate effect of silencing *14-3-3 ε* on the survival and reproduction of RWAs is still unclear and needs to be investigated in on-going experiments.

When comparing the two different experimental procedures by looking at the fecundity data, it appears that the control aphids would more readily produce nymphs when feeding on injected wheat leaves (only with Tris), rather than when feeding on artificial medium in a tube. This could be due to the latter being a less natural mode of feeding. At 48h of the injection trial, control aphid 4 was observed to be dead and control aphid 3 and 5 at 72h. A possible explanation for the latter could be that the aphids got stuck in the little bit of exposed glue of the custom-made cages and that this hindered their feeding abilities, but this could not be confirmed. Therefore, both methods have their drawbacks. Considering the results pertaining to the expression analysis, it seems that the method of subjecting aphids to feeding on artificial medium (containing siRNA) in custom-made tubes is more feasible and accurate. Although similar trends were observed, the custom-made aphid cages and injection of siRNA into wheat leaves appear to hinder the accuracy of the results. Nonetheless, as this method provides the aphids a more natural mode of feeding, alternative cage options need to be designed and tested to improve on the weaknesses and thus more precise results will be obtained.

4.5 References

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4.6 Appendix A

Diuraphis noxia COO2 gene, partial cds

GenBank: JN092369.1

FASTA Graphics

Go to:

```
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ACCESSION   JN092369
VERSION     JN092369.1  GI:388556077
KEYWORDS    .
SOURCE      Diuraphis noxia (Russian wheat aphid)
  ORGANISM  Diuraphis noxia
            Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Hexapoda; Insecta;
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REFERENCE   1  (bases 1 to 261)
  AUTHORS   Gong,L., Cui,F., Sheng,C., Lin,Z., Reeck,G., Xu,J. and Kang,L.
  TITLE     Polymorphism and methylation of four genes expressed in salivary
            glands of Russian wheat aphid (Homoptera: Aphididae)
  JOURNAL   J. Econ. Entomol. 105 (1), 232-241 (2012)
   PUBMED   22420276
REFERENCE   2  (bases 1 to 261)
  AUTHORS   Gong,L., Cui,F., Sheng,C.Z., Reeck,G., Xu,J.C. and Kang,L.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-JUN-2011) State Key Laboratory of Integrated Pest
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            Beichen West Road, Chaoyang District, Beijing 100101, P.R. China
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       61 acgtcggaca atttcagaga gacgtcggag gtgttcgaac gtatctggac aactccagag
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      181 atgagaagcc gactgttcaa agtgattgaa gcagctatgg atctgatgtc gaaatccaaa
      241 gacggagaat caatgaaaca g
//
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Figure A1: *Diuraphis noxia C002* GenBank record (JN092369.1).

Acyrthosiphon pisum 14-3-3 protein epsilon (14-3-3epsilon), mRNA

NCBI Reference Sequence: NM_001162004.2

FASTA Graphics

Go to:

```
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ACCESSION   NM_001162004 XM_001952260
VERSION     NM_001162004.2  GI:253735698
KEYWORDS    RefSeq.
SOURCE      Acyrthosiphon pisum (pea aphid)
  ORGANISM  Acyrthosiphon pisum
            Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Hexapoda; Insecta;
            Pterygota; Neoptera; Paraneoptera; Hemiptera; Sternorrhyncha;
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COMMENT     VALIDATED REFSEQ: This record has undergone validation or
            preliminary review. The reference sequence was derived from
            EX619839.1, AK341274.1, EX631448.1, CN764364.1, EX622245.1,
            FF330105.1 and EX623543.1.
            On Jul 11, 2009 this sequence version replaced gi:240849452.

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            RNAseq introns              :: single sample supports all introns
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Figure A2: *Acyrthosiphon pisum 14-3-3 protein epsilon* (NCBI Reference Sequence: NM_001162004.2).

Table A1: Relative expression values of *C002* and *14-3-3 ε* of SAM vs. SA1 at 0h using *L27* and *L32* as reference genes.

	R_{AVE}				SE			
Transcript	*C002*		*14-3-3 ε*		*C002*		*14-3-3 ε*	
Reference gene	*L27*	*L32*	*L27*	*L32*	*L27*	*L32*	*L27*	*L32*
SA1	1	1	1	1	0.15	0.15	0.07	0.07
SAM	2.92	3.5	2.23	2.63	0.23	0.28	0.17	0.2

Table A2: Student t-test p-values for SA1 vs. SAM at 0h obtained from SigmaPlot® to determine statistical significance of relative expression values for *C002* and *14-3-3 ε* using *L27* and *L32* as reference genes.

	C002		*14-3-3 ε*	
	L27	*L32*	*L27*	*L32*
p-value	1.4555e-3	2.2593e-3	1.4499e-3	1.455e-3

Table A3: Relative expression of *C002* in control aphids feeding on artificial medium compared to aphids feeding on artificial medium containing *C002* siRNA at different time points, with *L27* and *L32* utilised as reference genes (SE values also indicated).

	6h				24h				48h				72h			
	L27		*L32*		*L27*		*L32*		*L27*		*L32*		*L27*		*L32*	
	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE
Control Aphids	1.00	0.17	1.00	0.17	144.72	10.79	3.23	0.24	0.00	0.12	0.00	0.00	0.08	0.12	1.15	0.77
Test Aphids	1.48	0.10	0.67	0.04	15.05	2.34	1.68	0.26	1.21	1.94	0.89	0.56	0.44	1.82	2.22	0.54

Table A4: Student t-test p-values for control vs. *C002* siRNA exposed aphids at different time points obtained from SigmaPlot® to determine statistical significance of relative expression values for *C002* using *L27* and *L32* as reference genes.

	6h		24h		48h		72h	
	L27	*L32*	*L27*	*L32*	*L27*	*L32*	*L27*	*L32*
p value	8.11e-2	1.192e-1	2.967e-4	1.25e-2	1.104e-1	9.87e-2	1.67e-2	4.406e-1

Table A5: Relative expression values of *C002*, at different time points, in control aphids feeding on wheat leaves injected with Tris compared to aphids feeding on wheat leaves injected with Tris and *C002* siRNA, with *L27* and *L32* utilised as reference genes (SE values also indicated).

	6h				24h				48h				72h			
	L27		*L32*		*L27*		*L32*		*L27*		*L32*		*L27*		*L32*	
	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE
Control Aphids	1.00	1.61	1.00	1.61	6.10	11.90	7.43	14.51	4.54	0.55	14.42	1.74	0.83	0.27	1.24	0.41
Test Aphids	0.39	0.06	0.46	0.07	3.40	7.43	4.75	10.37	3.12	0.69	4.80	1.07	3.16	1.33	10.86	4.56

Table A6: Student t-test p-values for control vs. *C002* siRNA exposed aphids at different time points obtained from SigmaPlot® to determine statistical significance of relative expression values for *C002* using *L27* and *L32* as reference genes.

	6h		24h		48h		72h	
	L27	*L32*	*L27*	*L32*	*L27*	*L32*	*L27*	*L32*
p value	3.328e-1	3.5e-1	8.032e-1	8.884e-1	2.077e-1	9.3e-3	1.199e-1	7.48e-2

Table A7: Fecundity trial data for aphids feeding on an artificial diet or wheat leaves injected with siRNA. The amount of nymphs was counted daily at time intervals 24h - 72h. Numbers indicated in blue shows when the adult RWA was observed to be dead – either at 48h of 72h after exposure to treatment.

	Adult RWA no.	Feeding with artificial diet trial 24h	48h	72h	Total nymphs	Injection of siRNA into wheat trial 24h	48h	72h	Total nymphs
Control	1	0	1	1	2	0	1	2	3
	2	0	0	0	0	0	1	3	4
	3	0	0	0	0	1	1	1	3
	4	0	0	1	1	0	0	0	0
	5	0	0	0	0	0	0	0	0
	6	0	0	0	0	1	1	1	3
					3				13
C002	1	1	1	2	4	1	1	1	3
	2	0	0	0	0	0	0	0	0
	3	1	2	2	5	0	0	0	0
	4	0	0	0	0	1	1	3	5
	5	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	1	1	2
					9				10
14-3-3 ε	1	1	1	3	5	1	1	1	3
	2	0	0	0	0	1	3	3	5
	3	0	0	1	1	0	0	1	1
	4	0	0	2	2	1	1	1	3
	5	0	0	1	1	0	1	1	1
	6	0	0	1	1	1	1	1	3
					10				17

Table A8: Relative expression of *14-3-3 ε* in control aphids feeding on artificial medium compared to aphids feeding on artificial medium containing *14-3-3 ε* siRNA at different time points, with *L27* and *L32* utilised as reference genes (SE values also indicated).

	6h				24h				48h				72h			
	L27		*L32*		*L27*		*L32*		*L27*		*L32*		*L27*		*L32*	
	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE
Control Aphids	1.00	0.00	1.00	0.00	9.95	3.34	27.44	9.29	0.16	0.00	0.03	0.00	0.31	0.26	0.39	0.34
Test Aphids	3.31	0.11	3.55	0.11	2.99	0.40	2.71	0.36	0.55	0.05	1.14	0.10	0.98	0.44	2.13	0.97

Table A9: Student t-test p-values for control vs. *14-3-3 ε* siRNA exposed aphids at different time points obtained from SigmaPlot® to determine statistical significance of relative expression values for *14-3-3 ε* using *L27* and *L32* as reference genes.

	6h		24h		48h		72h	
	L27	*L32*	*L27*	*L32*	*L27*	*L32*	*L27*	*L32*
p value	7.156e-1	7.273e-1	2.04e-2	2.73e-03	8.243e-1	8.564e-1	5.953e-1	4.853e-1

Table A10: Relative expression values of *14-3-3 ε*, at different time points, in control aphids feeding on wheat leaves injected with Tris compared to aphids feeding on wheat leaves injected with Tris and *14-3-3 ε* siRNA, with *L27* and *L32* utilised as reference genes (SE values also indicated).

	6h				24h				48h				72h			
	L27		*L32*		*L27*		*L32*		*L27*		*L32*		*L27*		*L32*	
	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE	R_{AVE}	SE
Control Aphids	1.00	2.40	1.00	2.40	4.72	14.12	8.31	24.83	0.03	0.01	0.12	0.02	0.16	0.02	0.31	0.04
Test Aphids	1.58	1.92	0.85	1.03	1.08	0.76	1.53	1.08	0.51	0.59	2.52	2.93	0.48	0.40	0.99	0.83

Table A11: Student t-test p-values for control vs. *14-3-3 ε* siRNA exposed aphids at different time points obtained from SigmaPlot® to determine statistical significance of relative expression values for *14-3-3 ε* using *L27* and *L32* as reference genes.

	6h		24h		48h		72h	
	L27	*L32*	*L27*	*L32*	*L27*	*L32*	*L27*	*L32*
p value	9.529e-1	6.641e-1	2.66e-1	2.597e-1	2.042e-1	2.14e-1	2.466e-1	2.384e-1

Chapter 5:

Summary

5.1 Summary

The Russian wheat aphid, *Diuraphis noxia* (Kurdjumov, Hemiptera: Aphididae), also known as the Russian wheat aphid, is a damaging insect pest to important cereal crops like wheat and barley, causing significant losses in all major cereal production regions. Aphids are phytophagous insects possessing fine, needle-like stylets that are able to penetrate between plant cells, puncturing individual cells in the process, to ultimately establish a feeding site in a single phloem cell in the sieve element (Rao *et al*. 2013). Several studies have been suggesting that the salivary proteins released into the host plant during aphid feeding, seem to contain virulence factors, referred to as effectors, that hinders the host plant defences and enables colonisation (Musser *et al*. 2002; Will *et al*. 2007; Mutti *et al*. 2008; Bos *et al*. 2010; Stuart *et al*. 2012; Atamian *et al*. 2013; Pitino and Hogenhout 2013; Chaudhary *et al*., 2014; Elzinga *et al*. 2014; Guo *et al*. 2014; Rodriguez *et al*. 2014; Naessens *et al*. 2015; Wang *et al*. 2015). Although it's clear that saliva serves as a critical biochemical interface between aphids and their host plants, the physiological function and biochemical nature of the aphid salivary proteins is yet to be fully revealed and understood (Vandermoten *et al*. 2014). Two promising RWA salivary secretion proteins have been identified as potential effectors, namely C002 and 14-3-3 ε (Cloete 2015), but their role in aphid virulence still needs to be elucidated.

Up until now, the most effective means of RWA management has been the development and distribution of resistant wheat varieties in the most severely infested regions and secondly, the steady increase in native natural enemy populations. However, the rise in new RWA biotypes contributes to the significant challenges faced by the wheat producing industries. This causes RWA resistant wheat, employed as a long-term solution to RWA control, to no longer be as effective in regions subjected to new biotypes (Jankielsohn 2011). Additionally, the utilisation of natural enemies may require months or even years to effectively control pests (Knutson *et al*. 2014). Adverse weather conditions as well as modifications to crop

production practices can lead to a reduction in natural enemy populations, thus causing the recurrence of pests (Knutson *et al*. 2014). Producers then have to depend on insecticides to limit aphid damage and to reduce population numbers. Unfortunately, insecticides are not preventative and can kill predators and other beneficial insects (Knutson *et al*. 2014). Therefore, it is clear that a significant need exists for the establishment of an alternative and more effective RWA control strategy.

RNA interference (RNAi) has emerged as a prospective approach underlying the next generation of insect-resistant transgenic plants and is described as a gene silencing mechanism generated via double-stranded RNA (dsRNA) (Rodrigues & Figueira 2016). Successful delivery of dsRNA molecules to insects through ingestion has been reported. The aforementioned caused silencing of the expected essential target gene and resulted in death or influenced the fecundity of the target insect – thus controlling the pest (Huvenne & Smagghe 2009; Whyard *et al*. 2009).

To determine the role of *C002* and *14-3-3 ε* in the survival and reproduction of the RWA, it was attempted to establish a cell-based expression system using the Sf9 cell line. This system was going to be used for the *in vitro* expression and silencing of the aforementioned transcripts, but during the culturing thereof unforeseen contamination occurred. Efforts were made to verify and characterise the suspected viral contamination via SEM (Figure 3.5) and PCR analysis (Figure 3.6), but unfortunately were unsuccessful. Thus, it was decided to try and establish a RWA primary cell culture with isolated SAM embryos that could be utilised as a substitute cell-based expression system. Persistent bacterial contamination of the culture was encountered, despite making several adjustments to the protocol. Therefore, an alternative approach had to be followed to investigate whether *C002* and *14-3-3 ε* are important during RWA-wheat interactions.

In order to answer the main research question, the selected insect transcripts were silenced *in vivo* in the RWA using the RNAi tactic. Both *C002* and *14-3-3 ε* were subjected to

silencing using two different experimental setups. Firstly, the aphids were made to feed on artificial medium containing *C002* and *14-3-3 ε* siRNA, respectively. Secondly, aphids were exposed to wheat leaves injected with *C002* and *14-3-3 ε* siRNA, respectively. During both experiments aphids were sampled at 6h, 24h, 48h, and 72h after feeding was initiated and fecundity data was collected at 24h, 48h, and 72h to determine the effect of the silencing on the survival and reproduction of the aphids. RT-qPCR analysis, using *L27* and *L32* as reference genes, was performed to look at the relative expression levels and to verify whether successful silencing was achieved.

Firstly, the relative expression levels of *C002* and *14-3-3 ε* at 0h were compared between SAM, the most virulent RWA biotype, and SA1, the least virulent RWA biotype in South Africa. The results indicated that both transcripts had a higher relative expression in SAM than in SA1 (Table A1; Figure 4.6) and the observed differences were proven to be statistically significant as the p-values were all smaller than 0.05 ($p \leq 0.05$) (Table A2). Therefore, suggesting that *C002* and *14-3-3 ε* might play an important part in RWA virulence.

The results obtained from RT-qPCR analysis performed on sampled aphids from the feeding with artificial medium containing *C002* siRNA experiment indicated that successful silencing took effect at 24h after feeding was initiated (Figure 4.7; Table A3) and was verified as statistically significant by t-test results (Table A4). The differences in expression levels observed at 6h, 48h and 72h were not found to be statistically significant (p-values larger than 0.05) (Table A4). Thus, it suggests that transient silencing of *C002* is seen at 24h after initial siRNA exposure and thereafter the effect tapers off and expression levels return to normal. Similar RT-qPCR results were observed for the aphids feeding on *C002* siRNA injected wheat leaves. Although it seems that silencing is successful at 6-48h after initial siRNA exposure (Figure 4.9; Table A5), however the standard deviations are not ideal and suggests that the expression of this transcript differs greatly between the aphids within each group. Therefore, it can be concluded that the artificial diet experimental setup produces

more accurate results pertaining to transcript silencing. The fecundity data indicated that the silencing of *C002* during the leaf injection experiment hindered the reproduction of the aphids as it was seen that the control aphids produced more nymphs than the siRNA exposed aphids (Figure 4.10), thus corroborating the findings of Pitino *et al*. (2011). However, the inverse is observed for the artificial medium trial where more nymphs were produced by the siRNA exposed females (Figure 4.8). Dead aphids were seen at 48h and 72h during the leaf injection trial (Table A7), which could be a result of the successful silencing or due to aphids getting stuck in the bit of glue exposed in the aphid cage.

Results comparative to *C002* silencing were seen for the artificial diet trial pertaining to *14-3-3 ε*. The higher relative expression of *14-3-3 ε* observed at 24h in the control aphids when compared to the siRNA exposed aphids (Figure 4.11), were found to be statistically significant, while the differences in expression levels at the other time-points all displayed p values larger than 0.05 (Table A9) during the t-test analysis. Therefore, successful silencing of *14-3-3 ε* seemingly occurred at 24h after siRNA exposure. RT-qPCR analysis performed on aphids subjected to feeding on *14-3-3 ε* injected wheat leaves showed similar trends to the results of the artificial diet trials. This transcript had higher levels of expression in control aphids at 24h once more, while the inverse is visible at the other time-points (Figure 4.13). Unfortunately, yet again the standard deviations observed are quite high, indicating a lot of variation in the level of *14-3-3 ε* expression of individual aphids within the same group. Also, none of the differences were proven to be statistically significant after performing student t-tests (Table A11). The *14-3-3 ε* fecundity data produced quite surprising results as the total nymph production was significantly higher in the siRNA exposed aphids than in the control aphids during the artificial medium trial (Figure 4.12), while the siRNA treated aphids also gave birth to more nymphs when compared to control aphids during the leaf injection experiment (Figure 4.14). It seems that RNAi as a result of *14-3-3 ε* siRNA exposure might be slower acting than with *C002* siRNA. This is said as more nymphs were produced by the adult aphids exposed to *14-3-3 ε* siRNA (also at earlier time-points) and decreased levels of

C002 in the siRNA exposed aphids are seen as early as 6h after feeding is initiated. Furthermore, dead aphids are found as early as 48h after *C002* exposure, while they were only observed at 72h after *14-3-3 ε* exposure.

In conclusion; it is clear that successful silencing of both *C002* and *14-3-3 ε* were achieved at 24h after feeding commenced and that the transient silencing effect subsided thereafter. The fecundity data produced quite inconclusive results due to previously mentioned inadequacies and therefore an accurate and decisive conclusion cannot be drawn as to how the *C002* and *14-3-3 ε* silencing effects the survival and reproduction of the RWA. Both methods used for RNAi – the artificial diet trial and the injection of wheat leaves trial – have their drawbacks. After considering the RT-qPCR data, it appears as though the artificial diet trial produced more accurate and feasible results, while the custom-made aphid cages and the injection procedure of siRNA into wheat leaves deter the accuracy of the results obtained from that trial. Even so, the former method establishes a more natural mode of feeding for the aphids and consequently more optimal cages need to be designed and tested to produce precise results.

5.2 Reference list

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